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Patent application No.: PA 2002 00753

Date of filing: 16 May 2002

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Title: Expression of homologous sequences

IPC: C12N 7/01; A61K 39/275; C12N 15/40; C12N 15/863// A61K 39/285

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Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

25 April 2003

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PATENT- OG VAREMÆRKESTYRELSEN

Expression of homologous sequences

The present invention relates to a recombinant poxvirus capable of expressing two or more sequences, which derive from different variants of a microorganism, and which have between each other a homology of 50% or above in the coding sequence The invention further relates to a method preparing such recombinant poxvirus and the use of such recombinant poxvirus as medicament or vaccine Additionally, a method for affecting, preferably inducing, an immune response in a living animal, including a human, is provided

Background of the invention

Every living organism is constantly challenged by infectious or pathogenous agents such as bacteria, viruses, fungi or parasites The so-called immune system prevents the organism from permanent infections, diseases or intoxination caused by such agents

The immune system of a mammal can be divided into a specific and an unspecific part although both parts are closely cross-linked The unspecific immune response enables an immediate defense against a wide variety of pathogenic substances or infectious agents The specific immune response is raised after a lag phase, when the organism is challenged with a substance for the first time This specific immune response is mainly based on the production of antigen-specific antibodies and the generation of macrophages and lymphocytes, e g cytotoxic T-cells (CTL) The specific immune response is responsible for the fact that an individual who recovers from a

specific infection is protected against this specific infection but still is susceptible for other infectious diseases In general, a second infection with the same or a very similar infectious agent causes much milder symptoms or no symptoms at all This so-called immunity persists for a long time, in some cases even lifelong The underlying effect is often referred to as immunological memory, which can be used for vaccination proposes

With the term vaccination a method is described, where an individual is challenged with a harmless, partial or inactivated form of the infectious agent to affect, preferably induce, an immunological response in said individual, which leads to long lasting - if not lifelong - immunity against the specific infectious agent

The human smallpox disease is caused by the Variola virus Variola virus belongs to the family of *Poxviridae*, a large family of complex DNA viruses that replicate in the cytoplasma of vertebrate and invertebrate cells

The family of *Poxviridae* can be divided into the two subfamilies *Chordopoxvirinae* and *Entomopoxvirinae* based on vertebrate and insect host range The *Chordopoxvirinae* comprise beside others the genera of *Orthopoxviruses* and *Avipoxviruses* (Fields Virology, ed by Fields B N , Lippincott-Raven Publishers, 3rd edition 1996, ISBN 0-7817-0253-4, Chapter 83)

The genera of *Orthopoxviruses* comprises variola virus, the causative agent of human smallpox, and also other viruses with economical importance, e g camelpox, cowpox, sheeppox, goatpox, monkeypox and Vaccinia virus All members of this genus are genetically related and have similar morphology or host range Restriction endonuclease maps have even shown high sequence identity from up to 90%

between different members of the *Ortopoxviruses* (Mackett & Archard, [1979], J Gen Virol, 45 683-701)

Vaccinia virus (VV) is the name given to the agent that was used at least the last 100 years for the vaccination against smallpox It is not known whether VV is a new species derived from cowpox or variola virus by prolonged serial passages, the living representative of a now extinct virus or maybe a product of genetic recombination Additionally, in course of the VV history, many strains of Vaccinia have arisen These different strains demonstrate varying immunogenicity and are implicated to varying degrees with potential complications, the most serious of which is post-vaccinial encephalitis However, many of these strains were used for the vaccination against smallpox For example the strains NYCBOH, Western Reserve or Wyeth were used primarily in US, while the strain Ankara, Bern, Copenhagen, Lister and MVA were used for vaccination in Europe As a result of the worldwide vaccination program with these different strains of VV in 1980 the WHO finally declared the successful eradication of variola virus

Nowadays, VV is mainly a laboratory strain, but beside this is still considered as the prototype of *Ortopoxviruses*, which is also the reason why VV became one of the most intensively characterized viruses (Fields Virology, ed by Fields B N , Lippincott-Raven Publishers, 3rd edition 1996, ISBN 0-7817-0253-4, Chapter 83 and 84)

More recently, VV has become also a very useful and versatile mammalian expression vector With the aid of DNA recombination techniques Vaccinia based viral vectors were engineered comprising DNA sequences, which code for heterologous proteins, antigens or epitopes (Mackett, *et*

al [1982] P N A S USA 79, 7415-7419) In such case where a heterologous DNA sequence is integrated at a site in the virus genome, which is non-essential for the life cycle of the virus, it is possible for the newly produced recombinant VV to be infectious and, additionally, to express the integrated DNA sequence in the infected cell (EP 83 286)

The usefulness of recombinant VV, expressing e g Hepatitis B virus surface antigen (HBsAg), Influenza virus hemaglutinin (InfHA) or *Plasmodium knowlesi* sporozoite antigen, as live vaccines for the prophylaxis of infectious diseases has been demonstrated and reviewed (Smith, *et al* [1984] Biotechnology and Genetic Engineering Reviews 2, 383-407)

A further advantage of VV is the capacity to take up multiple foreign sequences, genes or antigens within a single VV genome (Smith & Moss [1983], Gene, 25(1) 21-28) Furthermore, it has been reported that it is possible to elicit immunity to a number of heterologous infectious diseases with a single inoculation of a polyvalent vaccine (Perkus *et al* , [1985], Science, Vol 229, 981-984)

One example of the expression of various antigens by a single VV is described by Bray *et al* It was shown that a recombinant VV, which is capable to express three different structural proteins of dengue virus serotype 4, namely the capsid (C), pre-membrane (pre-M), envelope (E) protein, and two non-structural proteins of dengue virus serotype 4, namely NS1 and NS2a, had the ability to protect mice against a homologous dengue virus serotype 4 challenge (Bray *et al* , [1989], Virology 2853-2856)

The dengue virus, with its four serotypes dengue virus serotype 1 (Den-1) to dengue virus serotype 4 (Den-4), is

one important member of the *Flavivirus* genus with respect to infections of humans Dengue virus infection produces diseases that range from flu-like symptoms to severe or fatal illness, dengue haemorrhagic fever (DHF) with shock syndrome (DSS) Dengue outbreaks continue to be a major public health problem in densely populated areas of the tropical and subtropical regions, where mosquito vectors are abundant

The concern over the spread of dengue infection and other diseases induced by mosquito-borne Flaviviruses in many parts of the world has resulted in more efforts being made towards the development of dengue vaccines, which could prevent both dengue fever (DF), and dengue hemorrhagic fever (DHF) and in vaccines useful to protect the vaccinated individual against infections induced by some or all mosquito-borne flaviviruses

While most cases of DF are manifested after the first infection by any of the four serotypes, a large percentage of DHF cases occur in subjects who are infected for the second time by a serotype which is different from the first infecting serotype of dengue virus These observations give rise to the hypothesis that sequential infection of an individual having antibodies against one dengue serotype by a different virus serotype at an appropriate interval may result in DHF in a certain number of cases

Accordingly, vaccination against one serotype leads not to a complete protection against dengue virus infection, but only against infection with the same dengue virus strain Even more important, a person vaccinated against one serotype, has an increased risk of developing severe complications such as dengue hemorrhagic fever when said

person is infected from a dengue virus strain of a different serotype

Thus, a multivalent vaccine that contains antigens from all four dengue virus serotypes is desired

So far it had been suggested to prepare multivalent vaccines by mixing a panel of recombinant VV, each VV encoding sequences of a different viruses (Moss, [1990] Immunology, 2, 317-327) However, such a multivalent vaccine comprises several disadvantages Firstly, it is cumbersome to generate several independent recombinant VV Beside the separated production processes, also quality control and quality assurance is highly time consuming Secondly, an infection with a mixture of recombinant viruses expressing different sequences always bears the risk that the infection event is not particularly well balanced The main risk is that only individual recombinants, but not all different recombinants comprised in the multivalent vaccine, will infect target cells One reason might be an uneven distribution of recombinant viruses Another reason might be interferences between the different recombinant viruses while infecting single cells Such interferences are known as phenomenon of superinfection In this case, only some antigens, but not all different antigens of the multivalent vaccine will finally be expressed from infected cells and, thus, presented to the immune system of a patient As a consequence, immune protection will be obtained only against some of the antigens, but is far from providing a complete immune protection against the various antigens presented or presentable by the multivalent vaccine

In the context of a vaccine against dengue virus infection the approach of a multivalent vaccine has the disadvantage that if the different sequences are expressed in different amounts or in an unpredictable manner, as it had been shown for the envelope protein of dengue virus 2 (Deuble *et al* , [1988], J Virol 65 2853), then such an vaccination is

highly risky for a patient An incomplete vaccination using a panel of recombinant Vaccinia viruses will only provide an immune protection against some, but not against all serotypes of dengue virus Unfortunately, in case of dengue infection an incomplete vaccination is extremely unacceptable, since it increases the risk of lethal complications such as dengue hemorrhagic fever

Object of the Invention

It is therefore an object of the present invention to provide a stable, effective and reliable vaccine against infectious diseases, which can be caused by more than one strain, clade, variant or serotype of said infectious disease causing microorganism

It is a further object of the present invention to provide a stable, effective and reliable vaccine against dengue virus infectious, which allows reliable vaccination against all dengue virus serotypes

Detailed description of the Invention

Although it is known that Vaccinia viruses (VV) may undergo homologous recombination of short homologous sequences and thereby may delete homologous sequences (Howley *et al* , [1996], Gene 172 233-237) the inventors provided recombinant poxviruses, which allow a stable insertion of several homologous sequences into their genome Such sequences may according to the present invention encode peptides, polypeptides, proteins Thus, it has been the achievement of the inventors of the present invention to provide for the first time a recombinant poxvirus

comprising in the genome two or more exogenous coding sequences, which are homologous to each other

The finding according to the present invention was particularly unexpected, since according to Howely et al already short sequences from up to 300 base pairs (bp) were sufficient to induce genomic rearrangement and deletion of homologous sequences in Vaccinia virus Having this in mind one would normally expect that longer sequences would induce recombination events with an even higher probability However, according to the present invention even sequences comprising complete homologous genes can be stably inserted into the genome of one poxvirus according to the present invention

A recombinant poxvirus according to the present invention comprises the relevant genetic information condensed in as less as one single infectious unit or - in other words - in one virus particle Accordingly, there is no risk of uneven infection and unbalanced expression of the different homologous sequences Thus, the recombinant poxvirus according to the present invention comprising and capable of expressing several closely, not to say closest, related genes or almost identical antigenic epitopes in one cell, is particularly advantageous for the generation of multivalent vaccines

This advantage is particularly interesting for the development of vaccines against diseases, which can be caused by several closely related strains or serotypes of a virus, like e g dengue virus A recombinant poxvirus comprising homologous genes of different dengue virus serotypes is described in example 1

According to the present invention the term "exogenous sequence" refers to a DNA sequence, which are in nature not

normally found associated with the poxvirus of the invention

In the context of the present invention the term "homologous genes" refers to coding sequences, which encode proteins, polypeptides, peptides or antigenic epitopes Homologous genes according to the present invention show minor variations in the nucleic acid sequence and/or the translated protein sequence, but regarding the functionality of the translated protein, peptide or epitope they fulfill the same tasks and show the same functional properties Homologous genes according to the present invention have a sequence homology between each other of above 50% in the coding sequence Additionally, homologous genes - as understood according to the present invention - derive from different but related sources or organisms

Two homologous genes or sequences according to the present invention have a homology of 50% to 100%, if their base pair sequence in the coding sequence of the gene is 50% to 100% identical Homology is normally determined by sequence comparison, which is preferably performed with suitable computer software, such as for example "Align" (Align, Plus for Windows, Version 3 0, Scientific and Educational Services) According to one embodiment of the present invention the homology in the coding sequences is preferably 70% to 80%, more preferably 80% to 90% or more preferably 90% to 100%

The homologous genes or sequences according to the present invention can be derived from any microorganism, such as any virus except the vector virus, any bacterium, any fungus or parasite Preferably the homologous genes or sequences derive from an infectious or pathogenic

microorganism and most preferably from different strains or clades, variants or serotypes of said microorganism

The terms "strain" or "clade" are technical terms, well known to the practitioner, referring to the taxonomy of microorganisms The taxonomic system classifies all so far characterised microorganism into the hierarchic order of *Families, Genera, species, strains* (Fields Virology, ed by Fields B N , Lippincott-Raven Publishers, 4th edition 2001) While the criteria for the members of *Family* is their phylogenetic relationship, a *Genera* comprises all members which share common characteristics and a *species* is defined as a polythetic class that constitutes a replicating lineage and occupies a particular ecological niche The term "strain" or "clade" describes a microorganism, i e virus, which shares the common characteristics, like basic morphology or genome structure and organisation, but varies in biological properties, like host range, tissue tropism, geographic distribution, attenuation or pathogenicity The term "variants" or "serotypes" further distinguishes between members of the same strain, also called subtypes, which due to minor genomic variations show individual infection spectra or antigenic properties

According to a further embodiment of the present invention the homologous genes or sequences are selected from viruses, preferably viruses, which belong to the genera of Flaviviruses, such as preferable - but not limited to - dengue virus, western nile virus or Japanese encephalitis virus, which belong to the genera of Retroviruses, such as preferable - but not limited to - Human Immunodeficiency Virus (HIV), which belong to the genera of Enteroviruses, such as preferable - but not limited to - Hand, Foot and Mouth disease, EV71, which belong to the genera of

Rotaviruses or which belong to the genera of Orthomyxoviruses, such as preferable - but not limited to- Influenza virus

According to a further preferred embodiment the homologous genes are selected from dengue virus genes, preferably C, more preferably PreM, further preferably NS1 and/or NS2, or further preferably E

According to still a further embodiment the homologous genes are selected from different HIV stains or clades Preferably the homologous genes are selected from the gag/pol coding sequence, more preferably from the env coding sequence or further preferably from a combination of structural and/or regulatory HIV coding sequences

The vector virus suitable for the present invention is selected from the group of poxviruses, which can be easily cultured in selected host cells, preferably avian host cells, but which are highly replication deficient or actually not replicating in humans or human cells

According to some preferred embodiments the poxvirus according to the present invention is selected from the canarypox viruses (Plotkin *et al* [1995] Dev Biol Stand vol 84 pp 165-170 Taylor *et al* [1995] Vaccine, Vol 13 No 6 pp 539-549), fowlpox viruses (Afonso *et al* [2000] J Virol, pp 3815-3831 Fields Virology, ed by Fields B N , Lippincott-Raven Publishers, 4th edition 2001, Chapter 85 page 2916), penguin pox viruses (Stannard *et al* [1998] J Gen Virol, 79, pp 1637-1649) or derivatives thereof As these viruses belong to the genera of Avipoxviruses they can be easily cultured and amplified in avian cells However, in mammalian or human cells they are replication defective, which means that no infectious progeny viruses will be produced

According to a further embodiment of the present invention an attenuated Vaccinia viruses is used for the generation of recombinant poxviruses comprising two or more homologous genes

One - not limiting - example is the highly attenuated and host range restricted Vaccinia strain, Modified Vaccinia Ankara (MVA) (Sutter, G *et al* [1994], Vaccine 12 1032-40) MVA has been generated by about 570 serial passages on chicken embryo fibroblasts of the Ankara strain of Vaccinia virus (CVA) (for review see Mayr, A , *et al* [1975], Infection 3, 6-14) As a consequence of these long-term passages CVA deleted about 31 kilobases of its genomic sequence The resulting virus strain, MVA, was described as highly host cell restricted (Meyer, H *et al* , J Gen Virol 72, 1031-1038 [1991]) A typical MVA strain is MVA-575 that has been deposited at the European Collection of Animal Cell Cultures under the deposition number ECACC V00120707

In another embodiment the MVA-Vero strain or a derivative thereof can be used according to the present invention The strain MVA-Vero has been deposited at the European Collection of Animal Cell Cultures under the deposition number ECACC 99101431 The safety of the MVA-Vero is reflected by biological, chemical and physical characteristics as described in the International Patent Application PCT/EP01/02703 In comparison to normal MVA, MVA-Vero has one additional genomic deletion

The term "derivatives" of a virus according to the present invention refers to progeny viruses showing the same characteristic features as the parent virus but showing differences in one or more parts of its genome

Still another embodiment according to the present invention uses MVA-BN MVA-BN has been deposited at the European Collection of Animal Cell Cultures with the deposition number ECACC V00083008 By using MVA-BN or a derivative thereof a particular safe virus vaccine is generated, since it has been shown that the MVA-BN virus is an extremely high attenuated virus, derived from Modified Vaccinia Ankara virus Therefore, in the preferred embodiment the invention concerns as a viral vector MVA-BN or derivatives thereof containing two or more homologous genes according to the present invention (Example 1) The term "derivative of MVA-BN" describes a virus, which has the same functional characteristics compared to MVA-BN The features of MVA-BN, the description of biological assays allowing the evaluation whether a MVA is MVA-BN and a derivative thereof and methods allowing the generation of MVA-BN or derivatives thereof are described in Example 2 One easy way to examine a functional characteristic of MVA-BN or derivatives thereof is its attenuation and lack of replication in human HaCat cells

In a recombinant poxvirus according to the present invention the expression of the exogenous sequences is controlled preferably by a poxviral transcriptional control element, more preferably by a MVA, canary pox, fowl pox, or penguin pox transcriptional control element or most preferably a Vaccinia virus promoter Poxviral transcriptional control elements according to the present invention comprise further every transcription control element functional in a poxviral system

The insertion of the exogenous sequences according to the present invention is preferably directed into a non-essential region of the virus genome Non-essential regions are e g loci or open reading frames (ORF) of poxviral

genes, which are non-essential for the poxviral life cycle Also intergenic regions, which describe the sequence between two ORF, are considered as non-essential regions according to the present invention In another embodiment of the invention, the exogenous sequences are inserted at a naturally occurring deletion site of the MVA genome (disclosed in PCT/EP96/02926 and incorporated herein by reference)

The orientation of the inserted DNA does not have an influence on the functionality or stability of the recombinant virus according the present invention

Since the recombinant poxvirus according to the invention is highly growth restricted and, thus, highly attenuated, it is ideal to treat a wide range of mammals including humans and even immune-compromised humans Hence, the present invention also provides a pharmaceutical composition and also a vaccine for inducing an immune response in a living animal body, including a human

The pharmaceutical composition may generally include one or more pharmaceutically acceptable and/or approved carriers, additives, antibiotics, preservatives, adjuvants, diluents and/or stabilizers Such auxiliary substances can be water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, or the like Suitable carriers are typically large, slowly metabolized molecules such as proteins, polysaccharides, polylactic acids, polyglycollic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, or the like

For the preparation of vaccines, the recombinant poxvirus according to the invention is converted into a physiologically acceptable form This can be done based on the experience in the preparation of poxvirus vaccines used

for vaccination against smallpox (as described by Stickl, H *et al* [1974] Dtsch med Wschr 99, 2386-2392) For example, the purified virus is stored at -80°C with a titre of 5x10E8 $TCID_{50}$/ml formulated in about 10mM Tris, 140 mM NaCl pH 7 4 For the preparation of vaccine shots, e g , 10E2-10E8 particles of the virus are lyophilized in 100 ml of phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule Alternatively, the vaccine shots can be produced by stepwise freeze-drying of the virus in a formulation This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone or other aids such as antioxidants or inert gas, stabilizers or recombinant proteins (e g human serum albumin) suitable for *in vivo* administration The glass ampoule is then sealed and can be stored between 4°C and room temperature for several months However, as long as no need exists the ampoule is stored preferably at temperatures below -20°C

For vaccination or therapy the lyophilisate can be dissolved in 0 1 to 0 5 ml of an aqueous solution, preferably physiological saline or Tris buffer, and administered either systemically or locally, i e parenteral, subcutaneous, intravenous, intramuscular, by scarification or any other path of administration know to the skilled practitioner The mode of administration, the dose and the number of administrations can be optimized by those skilled in the art in a known manner However, most commonly a patient is vaccinated with a second shot about one month to six weeks after the first vaccination shot

The recombinant virus according to the present invention is used for the introduction of the exogenous coding sequence into a target cell, said sequence being either homologous

or heterologous to the target cell The introduction of an exogenous coding sequence into a target cell may be used to produce *in vitro* proteins, polypeptides, peptides or antigenic epitopes Furthermore, the method for introduction of a homologous or of a heterologous sequence into cells may be applied for *in vitro* and *in vivo* therapy For *in vitro* therapy, isolated cells that have been previously *(ex vivo)* infected with the recombinant poxvirus according to the invention are administered to the living animal body for inducing an immune response For *in vivo* therapy, the recombinant poxvirus according to the invention is directly administered to the living animal body for inducing an immune response In this case, the cells surrounding the site of inoculation are directly infected *in vivo* by the virus or its recombinant according to the invention After infection these cells synthesis the proteins, peptides or antigenic epitopes, which are encoded in the exogenous coding sequences and, subsequently, present them or parts thereof on the cellular surface Specialized cells of the immune system recognize the presentation of such foreign proteins, peptides or epitopes and launch a specific immune response

Methods to obtain recombinant poxviruses or to insert exogenous coding sequences into a poxviral genome are well known to the person skilled in the art Additionally the method is described in the examples and can also be deduced or completed from the following references

- *Molecular Cloning,* A laboratory Manual Second Edition By J Sambrook, E F Fritsch and T Maniatis Cold Spring Harbor Laboratory Press 1989 Describes techniques and know how for standard molecular biology techniques such cloning of DNA, RNA isolation, western blot analysis, RT-PCR and PCR amplification techniques

- *Virology Methods Manual* Edited by Brian WJ Mahy and Hillar O Kangro Academic Press 1996 Describes techniques for the handling and manipulation of viruses
- *Molecular Virology* A Practical Approach Edited by AJ Davison and RM Elliott The Practical Approach Series IRL Press at Oxford University Press Oxford 1993 Chapter 9 Expression of genes by Vaccinia virus vectors
- *Current Protocols in Molecular Biology* Publisher John Wiley and Son Inc 1998 Chapter 16, section IV Expression of proteins in mammalian cells using Vaccinia viral vector Describes techniques and know-how for the handling, manipulation and genetic engineering of MVA

According to the present invention two different methods for the generation of the virus according to the present invention are preferred In one embodiment the exogenous sequences of interest (all cloned under the transcriptional control of a poxvirus expression control element into different vectors, which additionally comprise sequences capable of directing the insertion of the exogenous sequence to different parts of the poxviral genome and optionally selection or marker cassettes) will be transfected all together into a suitable cell culture cells Preferably Chicken Embryo Fibroblasts (CEF) are used Subsequently, the cells are infected with a poxvirus After homologous recombination occurred the recombinant viruses, comprising two or more of the exogenous sequences, can be isolated The use of selection or marker cassettes simplifies the identification and isolation of the recombinant poxviruses

In another embodiment of the method for generating recombinant poxviruses according to the present invention a first plasmid vector, comprising an exogenous sequence with the gene under the transcriptional control of a poxvirus

expression control element, is transfected into suitable cell culture cells Preferably Chicken Embryo Fibroblasts (CEF) are used Beside the exogenous sequence the plasmid vector comprises also sequences capable of directing the insertion of the exogenous sequence to selected part in the poxviral genome In general such sequences are homologous to the sequences flanking the selected insertion site in the poxviral genome Optionally, the plasmid vector contains also a cassette comprising a marker and/or selection gene operably linked to a poxviral promoter The skilled practitioner knows several suitable marker or selection genes, beneath these are e g the genes encoding the Green Fluorescent Protein, ß-Galactosidase or other color markers, neomycin or other antibiotic resistance, phosphoribosyltransferase or other selection marker After infection as described above and homologous recombination the recombinant poxvirus can be isolated The use of selection or marker cassettes simplifies the identification and isolation of the recombinant poxvirus However, a recombinant poxvirus can also be identified by PCR technology

In the second round of transfection and infection a second vector and the recombinant poxvirus obtained in the first round is used The second vector differs from the first vector in the sequences directing the recombination of the exogenous sequence into the poxviral genome, since a different insertion site will be used Additionally, it differs in the exogenous sequence, which comprises a gene homologous to the gene from the first vector After homologous recombination occurred the recombinant virus, comprising two homologous genes in different insertion sites can be isolated The use of selection or marker cassettes simplifies the identification and isolation of

the recombinant poxvirus To introduce more than two homologous genes into the recombinant vector, the steps of transfection and infection are repeated always using for transfection a further vector comprising a further homologous gene and for the infection the recombinant virus isolated in the previous steps

The invention further provides a kit comprising two or more plasmid vector constructs capable of directing the integration of expressable genes into the poxvirus genome Beside a suitable cloning site such plasmid vectors comprise sequences capable of directing the insertion of the exogenous sequence to selected parts in the poxviral genome Optionally, such vectors comprise selection or marker gene cassettes The kit further comprises means and instructions to select viruses, which are recombinant for one or several of the homologous genes and optionally a selection or marker gene, inserted via said vector constructs

According to another further embodiment the invention includes DNA sequences or parts thereof derived from or homologous to the recombinant poxvirus of the present invention Such sequences comprise at least part of the exogenous sequence comprising at least a fragment of one of the homologous gene according to the present invention and at least a fragment of the genomic poxvirus sequence according to the present invention, said genomic poxvirus sequences preferably flanking the exogenous sequence

Such DNA sequences can be used to identify or isolate the virus or its derivatives, e g by using them to generate PCR-primers, hybridization probes or in array technologies

Summary of the Invention

The invention *inter alia* comprises the following, alone or in combination

A recombinant poxvirus comprising at least two heterologous genes, wherein each of the heterologous genes is inserted into a different insertion site of the viral genome and wherein the heterologous genes are homologous among each other,

the recombinant poxvirus as above, wherein the homologous genes have a homology of at least 50% in the coding sequence,

the recombinant poxvirus as above, wherein the homologous genes are derived from a microorganism, preferably from a pathogenic and/or infectious bacterium, fungus or virus,

the recombinant poxvirus as above, wherein the homologous genes are derived from two or more members of different *strains, clades, variants and/or serotypes of* the microorganism, bacterium, fungus or virus,

the recombinant poxvirus as above, wherein the microorganism is a flavivirus, a retrovirus, an enterovirus or a rotavirus,

the recombinant poxvirus as above, wherein the flavivirus is a dengue virus and the homologous genes are selected from the C, PreM, NS1, NS2 or E sequences of different dengue viruses,

the recombinant poxvirus as any above, wherein the poxvirus is replication deficient and/or not replicating in human cells,

the recombinant poxvirus as any above, wherein the poxvirus is selected from Fowlpox viruses or derivatives thereof, Canarypox viruses or derivatives thereof, or attenuated Vaccinia viruses or derivatives thereof,

the recombinant poxvirus as above, wherein the attenuated Vaccinia virus is Modified Vaccinia Ankara (MVA), MVA-Vero, MVA-BN or derivatives thereof,

the recombinant poxvirus as any above, wherein the insertion sites are selected from naturally occurring deletions in the pox virus genome, non-essential gene loci and/or intergenic regions,

the recombinant poxvirus as any above as medicament or vaccine,

vaccine comprising the recombinant poxvirus as any above,

pharmaceutical composition comprising the recombinant poxvirus as any above and a pharmaceutically acceptable carrier, diluent, adjuvant and/or additive,

the recombinant poxvirus as any above, the vaccine as above or the composition as above as drug for affecting, preferably inducing, an immune response of a living animal, including a human,

use of the recombinant poxvirus as any above, the vaccine as above or the composition as above for the preparation of a medicament for affecting, preferably inducing, an immune response of a living animal, including a human,

method for affecting, preferably inducing, an immune response in a living animal, including a human, comprising administering a therapeutically effective amount of the recombinant pox virus as any above, the vaccine as above or the composition as above to the animal or human to be treated,

method for producing a recombinant poxvirus as any above comprising the steps of

(i) transfecting cells with a first vector construct comprising an exogenous sequence having under the transcriptional control of a poxviral expression control element a gene encoding at least one protein or parts thereof, at least one polypeptide or peptide and/or at least one antigenic epitope and further comprising genomic poxvirus sequences capable of directing the integration of the exogenous sequence to an insertion site into a poxvirus genome,

(ii) infecting the transfected cells from (i) with a poxvirus,

(iii) identifying, isolating and optionally purifying a recombinant poxvirus,

(iv) repetition of steps (i) - (iii) with each vector construct, wherein the additional vector construct comprises an exogenous sequence having under the transcriptional control of a poxviral expression control element a gene, which is homologous to the gene of the first vector, wherein for infecting according to step (ii) the recombinant poxvirus obtained from the previous steps (i) - (iii) is used,

(v) isolating the recombinant poxvirus,

a kit comprising

(i) two or more vector constructs,

wherein each vector construct comprises an exogenous sequence having under the transcriptional control of a poxviral expression control element a gene encoding at least one protein or parts thereof, at least one polypeptide or peptide and/or at least one antigenic epitope, wherein the genes comprised in the different vectors are homologous genes,

wherein each vector construct is capable of directing the integration of the exogenous sequence into a different insertion site of the poxviral genome, and

(ii) means for identifying and/or selecting recombinant poxviruses, which have incorporated said exogenous sequences at different insertion sites into their genome,

DNA sequence or part thereof derived from or homologous to the recombinant poxvirus as any above, wherein the DNA sequence comprises at least part of the exogenous sequence comprising the homologous gene encoding at least one protein or parts thereof, at least one polypeptide or peptide and/or at least one antigenic epitope and at least part of the genomic poxvirus sequence of the recombinant poxvirus as any above, said genomic poxvirus sequences preferably flanking the exogenous sequence,

use of the DNA as above for detecting cells infected with a recombinant poxvirus as any above and/or identifying a recombinant poxvirus as any above,

Short Description of the Figures

Figure 1: Sequence comparison of the PrM gene of dengue virus serotype 1 to 4

Figure 2: Schematic presentation of the insertion sites of the four PrM genes (serotype 1 to 4) in the MVA genome

Figure 3 to 10: Insertion plasmid vector constructs indicating the name of the vector, its size, a variety of restriction enzyme sites and the localization of the sequences of interest such as

PrM = gene of dengue virus, number indicating from which of the four serotype it derives AmpR = ampicillin resistance gene, F1 = flanking sequence 1, F2 = flanking sequence 2,

dA = deletion A, dE = deletion E, d2 = deletion 2, I4L = intergenic region I4L, rpt = repetition of flanking sequence, P = poxvirus promoter, pr7 5 = Vaccinia promoter 7 5, NPT II = neomycin resistance gene, EGFP = enhanced green florescence protein gene, hbfp = humanized blue florescence protein gene, Ecogpt = guanosinphosphoribosyl-transferase gene

Figure 11 PCR verification of the vector cloning strategies of three different insertion vectors (pBN49, pBN50, pBN40, pBN39) Each of the plasmids was tested with 4 different PCR primer combinations Each combination is specific for one distinct PrM sequence integrated into one distinct insertion site

Figure 12 PCR verification of the recombinant poxvirus incorporating two or more homologous genes While in the upper part of the gel the different PCR results of the recombinant virus are shown, the lower part provides the results of the same PCR reactions of the control plasmids as indicated The plasmid containing the homologous sequences is named pBN39, pBN49 or pBN50 PrM stands for the inserted genes of dengue virus, wherein the numbers indicate from which of the four serotype it derives dA = deletion A, dE = deletion E, d2 = deletion 2, I4L = intergenic region I4L describes the insertion site of the exogenous DNA

The following examples will further illustrate the present invention It will be well understood by a person skilled in the art that the provided examples in no way may be interpreted in a way that limits the applicability of the technology provided by the present invention to this examples

Example 1

Insertion vectors

Insertion vector for deletion A

For the insertion of exogenous sequences into the MVA genome at the so-called deletion A or deletion 1 respectively, corresponding to the genome position 7608-7609, a plasmid vector was constructed, which comprises about 600 bp of the flanking sequences adjacent to the deletion site A To isolate the flanking sequences from the genomic MVA-BN DNA suitable PCR primers can be designed with suitable computer software (DNAsis, Hitashi software engeneering, San Bruno, USA) Such primers comprise extensions with restriction enzyme sites, which will be used to clone the flanking sequences into a vector plasmid In between these flanking sequences, a selection gene cassette is introduced, e g a NPT II gene (neomycin resistance) under the transcriptional control of a poxviral promoter Additionally, there is a cloning site for the insertion of additional genes or exogenous sequences to be inserted into deletion site A One such vector construct according to the present invention is disclosed in Figure 3 (pBNX10)

Insertion vector for deletion E

For the insertion of exogenous sequences into the MVA genome at the so-called deletion E or deletion 4 respectively, corresponding with the genome position 170480-170481, a vector was constructed, which comprises about 600 bp of the flanking sequences adjacent to the deletion site E The vector is designed and constructed like described above In between the flanking sequences is

located an EGPF gene (green fluorescing protein, Clonetech) under the transcriptional control of a poxviral promoter Additionally, there is a cloning site for the insertion of additional genes or sequences to be inserted into deletion site A One such vector construct according to the present invention is disclosed in Figure 4 (pBNX32)

Insertion vector for deletion 2

For the insertion of exogenous sequences into the MVA genome at the so-called deletion 2, corresponding with the genome position 20718-20719, a vector was constructed, which comprises about 600 bp of the flanking sequences adjacent to the deletion site 2 The vector is designed and constructed like described above In between the flanking sequences is located an hbfp gene (humanized blue fluorescing protein, Pavalkis GN et al) under the transcriptional control of a poxviral promoter Additionally, there is a cloning site for the insertion of additional genes or sequences to be inserted into deletion site 2 One such vector construct according to the present invention is disclosed in Figure 5 (pBNX36)

Insertion vector for intergenetic region, I4L

For the insertion of exogenous sequences in the intergenetic region, between the ORF I3L and I4L, corresponding to the genome position 56760, a vector was constructed, which comprises about 600 bp of the flanking sequences adjacent to the intergenetic region at the I4L locus The vector is designed and constructed like described above In between the flanking sequences is located an *Ecogpt* gene (or *gpt* stands for phosphoribosyl-transferase gene isolated from *E coli*) under the transcriptional control of a poxviral promoter

Additionally, there is a cloning site for the insertion of additional genes or sequences to be inserted into the intergenetic region after the I4L ORF One such vector construct according to the present invention is disclosed in Figure 6 (pBNX39)

Construction of recombinant poxvirus comprising several homologous genes integrated in its genome.

Insertion vectors

For the insertion of the four PrM genes of the four serotypes of dengue virus in the MVA genome four independent recombination vectors were used

These vectors contain - as described in details above - sequences homologous to the MVA genome for targeting insertion by homologous recombination Additionally each vector contains a selection- or reporter gene cassette

The PrM sequences of the four dengue virus serotypes (for sequence comparison see Figure 1) where synthetically made by oligo annealing and PCR amplification The PrM sequences were cloned downstream of poxvirus promoter elements to form an expression cassette This expression cassette was then cloned into the cloning site of the relevant insertion vector constructs

As result the insertion vector construct for deletion A contained the PrM gene of dengue virus serotype 2 (Figure 7 - pBN39) the insertion vector construct for deletion 2 contained the PrM gene of dengue virus serotype 1 (Figure 8 - pBN49) the insertion vector construct for intergenic region I4L contained the PrM gene of dengue virus serotype 3 (Figure 9 - pBN50) the insertion vector construct for deletion E contained the PrM gene of dengue virus serotype 4 (Figure 10 - pBN40)

PCR verification of the insertion vectors

For verification of the cloning strategies PCR assays were performed For these PCR assays the selected primer pairs are a combination of a primer specifically binding to the specific flanking sequence relative to the insertion site and a second primer specifically binding to one of the highly homologous dengue virus PrM genes

The insertion vector for deletion A containing the PrM gene of dengue virus serotype 2 was screened with the Primers oBN93 (CGCGGATCCATGCTGAACATCTTGAACAGGAGACGCAGA SEQ ID NO 1) and oBN477 (CATGATAAGAGATTGTATCAG SEQ ID NO 2)

The insertion vector for deletion 2 containing the PrM gene of dengue virus serotype 1 was screened with the Primers oBN194
(ATGTTGAACATAATGAACAGGAGGAAAAGATCTGTGACCATGCTCCTCATGCTGCTGC CCACAGCCCTGGCGTTCCATCT SEQ ID NO 3) and oBN476 (GATTTTGCTATTCAGTGGACTGGATG SEQ ID NO 4)

The insertion vector for intergenic region I4L containing the PrM gene of dengue virus serotype 3 was screened with the Primers oBN255 (CCTTAATCGAATTCTCATGTCATGGATGGGGTAACCAGCATTAATAGT SEQ ID NO 5) and oBN479 (GCTCCCATTCAATTCACATTGG SEQ ID NO 6)

The insertion vector for deletion E containing the PrM gene of dengue virus serotype 4 was screened with the Primers oBN210
(ATCCCATTCCTGAATGTGGTGTTAAAGCTACTGAGCGCTTCTCTCGTCTCCGTTCTCC GCTCTGGGTGCATGTCCCATAC SEQ ID NO 7) and oBN478 (GTACATGGATGATATAGATATG SEQ ID NO 8)

PCR experiments are performed in a Thermal cycler GeneAmp 9700 (Perkin Elmer) using the DNA Polymerase Kit (Qiagen) containing 10x PCR buffer, $MgCl_2$ buffer and *Taq* DNA polymerase (Roche, Cat no 201205) or equivalent In

general the PCR reactions were prepared with a total reaction volume of 50 µl containing 45 µl mastermix, the sample DNA and DdH_2O as required The mastermix should be prepared with 30 75 µl DdH_2O, 5 µl 10x buffer, 1 µl DNTP-mix (10 mM each), 2 5 µl of each primer (5 pmol/µl), 3 µl $MgCl_2$ (25 mM) and 0 25 µl Taq-polymerase (5 U/µl)

The amplification was performed using the following programme

1)	Denaturation	4 min	94°C
2)	30 Cycles		
	Denaturation	30 sec	94°C
	Annealing	30 sec	55°C
	Elongation	1-3 min	72°C
3)	Elongation	7 min	72°C
4)	Store		4°C

Based on the size of the inserted gene the elongation time should at least be 1min/kb

PCR results are shown in Figure 11

The primer combination oBN194/oBN476 is specific for deletion 2 and PrM1 as insert The expected PCR fragment of plasmid pBN49 has a size of 678 bp (shown in lane 3, upper part of the gel)

The primer combination oBN255/oBN479 is specific for intergenic region I4L and PrM3 as insert The expected PCR fragment of plasmid pBN50 has a size of 825 bp (shown in lane 9, upper part of the gel)

The primer combination oBN210/oBN478 is specific for deletion E and PrM4 as insert The expected PCR fragment of plasmid pBN40 has a size of 607 bp (shown in lane 5, lower part of the gel)

The primer combination oBN93/oBN477 is specific for deletion A and PrM2 as insert The expected PCR fragment of plasmid pBN39 has a size of 636 bp (shown in lane 11, lower part of the gel)

Generation of the recombinant MVA via homologous recombination

For expression of foreign genes by a recombinant MVA these genes have to be inserted into the viral genome by a process called homologous recombination For that purpose the foreign gene of interest had been cloned into an insertion vector, as described above This vector has to be transfected after infection of cells with MVA-BN The recombination will take place in the cellular cytoplasm of the infected and transfected cells With help of the selection and/or reporter cassette, which is also contained in the insertion vector, cells comprising recombinant viruses are identified and isolated

Homologous recombination

For homologous recombination BHK (Baby hamster kidney) cells or CEF (primary chicken embryo fibroblasts) are seeded in 6 well plates using DMEM (Dulbecco's Modified Eagles Medium, Gibco BRL) + 10% fetal calf serum (FCS) or VP-SFM (Gibco BRL) + 4mmol/l L-Glutamine for a serum free production process

Cells need to be still in the growing phase and therefore should reach 60-80% confluence on the day of transfection Cells were counted before seeding, as the number of cells has to be known for determination of the multiplicity of infection (moi) for infection

For the infection the MVA stock is diluted in DMEM/FCS or VP-SFM/L-Glutamine so that 500 µl dilution contain an

appropriate amount of virus that will give a moi of 0 01 Cells are assumed to be divided once after seeding The medium is removed from cells and cells are infected with 500µl of diluted virus for 1 hour rocking at room temperature The inoculum is removed and cells are washed with DMEM/VP-SFM Infected cells are left in 1 6ml DMEM/FCS and VP-SFM/L- Glutamine respectively while setting up transfection reaction (Qiagen Effectene Kit)

For the transfection the "Effectene" transfection kit (Qiagen) is used A transfection mix is prepared of 1-2 µg of linearized insertion vector (total amount for multiple transfection) with buffer EC to give a final volume of 100 µl Add 3 2 µl Enhancer, vortex and incubate at room temperature for 5 min Then, 10 µl of Effectene are added after vortexing stock tube and the solution is mixed thoroughly by vortexing and incubated at room temperature for 10 min 600 µl of DMEM/FCS and VP-SFM/L-Glutamine respectively, are added, mixed and subsequently, the whole transfection mix is added to the cells, which are already covered with medium Gently the dish is rocked to mix the transfection reaction Incubation takes place at 37°C with 5%CO_2 over night The next day the medium is removed and replaced with fresh DMEM/FCS or VP-SFM/L-Glutamine Incubation is continued until day 3

For harvesting the cells are scraped into medium, then the cell suspension is transferred to an adequate tube and frozen at -20°C for short-term storage or at -80°C for long term storage

Insertion of PrM4 into MVA

In a first round, cells were infected with MVA-BN according to the above-described protocol and were additionally transfected with insertion vector pBN40 containing the PrM

gene of dengue virus serotype 4 and as reporter gene the EGPF gene Since the transfected vector contains a reporter gene, EGFP, the synthesized protein is detectable latest on day three in cells infected with a recombinant virus Resulting recombinant viruses have to be purified by plaque purification

For plaque purification infected cells (fluorescing or stained) are isolated with a pipet tip, resuspended and aspirated in 200µl PBS or medium Then a fresh culture dish containing about 10E6 cells is infected with 100µl of the resuspended plaques After 48h cells are taken up in 300µl PBS DNA is extracted from suspension and screened with PCR analysis A clone that shows the expected bands is chosen and fresh 6-well plates are infected with different amounts of this virus Overlaying the wells with 1% agarose avoids further spreading of virus After 48h infected cells comprising a recombinant virus clone are isolated

This procedure is repeated until no wild-type MVA-BN can be detected in the PCR analysis

After 4 rounds of plaque purification recombinant viruses, MVA-PrM4, were identified by PCR assays using a primer pair selectively amplifying the expected insertion (oBN210 and oBN478, as described above) and as control a primer pair specifically recognizing the insertion site deletion E (oBN453 GTTGAAGGATTCACTTCCGTGGA, SEQ ID NO 9 and oBN454 GCATTCACAGATTCTATTGTGAGTC, SEQ ID NO 10)

Insertion of PrM2 into MVA-PrM4

Cells were infected with MVA-PrM4 according to the above described protocol and were additionally transfected with insertion vector pBN39 containing the PrM gene of dengue

virus serotype 2 and as selection gene the NPT II, a neomycin resistance gene For purification of recombinant MVA expressing an antibiotic resistance gene three rounds of virus amplification under selective conditions before plaque purification are recommended For neomycinphosphotransferase selection G418 is added to the medium G418 is a derivative of neomycin and inhibits the protein-biosynthesis by interference with the action of the ribosomes NPT gene activity inactivates G418 by phosphorylation

After 16 rounds of plaque purification under neomycin selection recombinant viruses, MVA-PrM4/PrM2, were identified by PCR assays using a primer pair selectively amplifying the expected insertion (oBN93 and oBN477, as described above) and as control a primer pair specifically recognizing the insertion site deletion A (oBN477 as described above) and oBN452 GTTTCATCAGAAATGACTCCATGAAA, SEQ ID NO 11) Additionally also insertion of PrM4 into deletion E is verified with the primer pairs oBN210 - oBN478 and oBN453 - oBN454

Insertion of PrM1 into MVA

In a first round, cells were infected with MVA-BN according to the above described protocol and were additionally transfected with insertion vector pBN49 containing the PrM gene of dengue virus serotype 1 and as reporter gene the hbfp, the gene for humanized blue fluorescing protein The synthesized hbfp protein is detectable on day three in cells infected with a recombinant virus Resulting recombinant viruses were purified by plaque purification

After 10 rounds of plaque purification recombinant viruses, MVA-PrM1, were identified by PCR assays using a primer pair selectively amplifying the expected insertion (oBN194 and

oBN476, as described above) and as control a primer pair specifically recognizing the insertion site deletion 2 (oBN54 CGGGGTACCCGACGAACAAGGAACTGTAGCAGAGGCATC, SEQ ID NO 12 and oBN56 AACTGCAGTTGTTCGTATGTCATAAATTCTTTAATTAT, SEQ ID NO 13)

Insertion of PrM3 into MVA

In a first round, cells were infected with MVA-BN according to the above described protocol and were additionally transfected with insertion vector pBN50 containing the PrM gene of dengue virus serotype 3 and as reporter gene the *Ecogpt* gene (*Ecogpt* or shortened to *gpt* stands for phosphoribosyltransferase gene) Resulting recombinant viruses were purified by 3 rounds of plaque purification under phosphribosyltransferase metabolism selection by addition of mycophenolc acid, xanthin and hypoxanthin Mycphenolic acid (MPA) inhibits inosine monophosphate dehydrogenase and results in blockage of purine synthesis and inhibition of viral replication in most cell lines This blockage can be overcome by expressing *Ecogpt* from a constitutive promoter and providing the substrates xanthine and hypoxanthine

Resulting recombinant viruses, MVA-PrM3, were identified by PCR assays using a primer pair selectively amplifying the expected insertion (oBN255 and oBN479, as described above) and as control a primer pair specifically recognizing the insertion site I4L (oBN499 CAACTCTCTTCTTGATTACC, SEQ ID NO 14 and oBN500 CGATCAAAGTCAATCTATG, SEQ ID NO 15)

Coinfection of MVA-PrM1 and MVA-PrM3

The cells were infected with equal amounts of MVA-PrM1 and MVA-PrM3 according to the above protocol After 3 rounds of plaque purification under phosphribosyltransferase

metabolism selection of blue fluorescing clones of recombinant viruses were analyzed by PCR using the primer pairs (oBN255 and oBN479 oBN499 and oBN500 oBN194 and oBN476 oBN54 and oBN56 as described above) Resulting recombinant viruses were designated MVA-PrM1/PrM3

Coinfection of MVA-PrM1/PrM3 and MVA-PrM2/PrM4

The cells were infected with equal amounts of MVA-PrM1/PrM3 and MVA-PrM2/PrM4 according to the above protocol Plaque purification was performed under phosphribosyltransferase metabolism and neomycin selection Recombinant viruses inducing a green and blue flourescence were isolated and analyzed by PCR using the primer pairs (oBN255 and oBN479 oBN499 and oBN500 oBN194 and oBN476 oBN54 and oBN56 oBN93 and oBN477 oBN477 and oBN452 oBN210 and oBN478 oBN453 and oBN454 as described above)

The PCR analysis of the recombinant virus (Clone 20) comprising all four PrM genes is shown in Figure 12 While in the upper part of the gel the different PCR results of the recombinant virus are shown, the lower part provides the results of the same PCR reactions of the control plasmids (as indicated) Lane 1, 10 and 11 show a 1kb and a 100bp molecular marker

The primer combination oBN210/oBN478 is specific for deletion E and PrM4 as insert The expected PCR fragment of the recombinant virus and the plasmid pBN40 has a size of 607 bp (shown in lane 2)

The primer combination oBN453/oBN454 is specific for deletion E The expected PCR fragment of the recombinant virus is 2 7 kb, the expected band of the wild-type virus 2 3 kb (shown in lane 3) Also in the upper part of the gel a band specific for a wild-type virus can be identified

This means that the recombinant virus preparation is not yet completely free of wild-type virus Further plaque purification is necessary

The primer combination oBN93/oBN477 is specific for deletion A and PrM2 as insert The expected PCR fragment of the recombinant virus and the plasmid pBN39 has a size of 636 bp (shown in lane 4)

The primer combination oBN477/oBN452 is specific for deletion A The expected PCR fragment of the recombinant virus is 4 1 kb, the expected band of the wild-type virus 2 7 kb (shown in lane 5) In the upper part of the gel a band specific for a wild-type virus can be identified

The primer combination oBN255/oBN479 is specific for intergenic region I4L and PrM3 as insert The expected PCR fragment of the recombinant virus and the plasmid pBN50 has a size of 825 bp (shown in lane 6)

The primer combination oBN499/oBN500 is specific for the intergenic region of I4L The expected PCR fragment of the recombinant virus is 1 0 kb, the expected band of the wild-type virus 0 3 kb (shown in lane 7)

The primer combination oBN194/oBN476 is specific for deletion 2 and PrM1 as insert The expected PCR fragment of the recombinant virus and the plasmid pBN49 has a size of 678 bp (shown in lane 8)

The primer combination oBN54/oBN56 is specific for deletion 2 The expected PCR fragment of the recombinant virus is 1 6 kb, the expected band of the wild-type virus 0 9 kb (shown in lane 9) In the upper part of the gel a band specific for a wild-type virus can be identified

Alternatively, several strategies are conceivable like purifying a virus with one insertion up to an empty-vector-

virus free state and following insertion of the next vector then by homologous recombination Alternatively one can produce 4 different viruses, coinfect cells with all four viruses and screen for a recombinant

Improvements can also be achieved with new recombination vectors, which contain further selection- or resistance markers

Example 2

Growth kinetics of the different MVA vector viruses in selected cell lines, replication in vivo and immunological data

As pointed out in the description section the exogenous coding sequence according to the present invention is inserted in the different MVA poxviral vector or derivatives thereof The following example describes such MVA in more detail The disclosed examples allow the person skilled in the art to determine the different MVA and, particularly their derivatives

1 Growth kinetics in cell lines

To characterize MVA-BN the growth kinetics of this strain were compared to those of other MVA strains, which have already been characterized

The experiment was done by comparing the growth kinetics of the following viruses in the subsequently listed primary cells and cell lines

1) MVA-BN (Virus stock #23, 18 02 99 crude, titrated at 2,0 x 10^7 $TCID_{50}$/ml) (ECACC V00083008)

ii) MVA as characterized by Altenburger (US patent 5,185,146) and further referred to as MVA-HLR (deposited at CNCM, France deposition number I-721)

iii) MVA (passage 575) as characterized by Anton Mayr (Mayr, A , *et al* [1975] Infection 3 6-14) and further referred to as MVA (ECACC V00120707)

iv) MVA-Vero as characterized in the International Patent Application PCT/EP01/02703 (ECACC 99101431)

The used primary cells and cell lines were

1 CEF Chicken embryo fibroblasts (freshly prepared from SPF eggs)

2 HeLa Human cervix adeocarcinoma (epithelial), ATCC No CCL-2

3 143B Human bone osteosarcoma TK-, ECACC No 91112502

4 HaCaT Human keratinocyte cell line, Boukamp *et al* 1988, J Cell Biol 106(3) 761-771

5 BHK Baby hamster kidney, ECACC 85011433

6 Vero African green monkey kidney fibroblasts, ECACC 85020299

7 CV1 African green monkey kidney fibroblasts, ECACC 87032605

For infection the different cells were seeded into 6-well-plates at a concentration of 5 x 10^5 cells/well and incubated over night at 37°C, 5% CO_2 in DMEM (Gibco, Cat No 61965-026) plus 2% FCS Cell culture medium was removed and cells were infected at approximately moi 0 05 for one hour at 37°C, 5% CO_2 (for infection it is assumed that cell numbers doubled over night) The amount of virus

used for each infection of the different cell types was 5 X 10^4 $TCID_{50}$ and this will be referred to as Input Cells were then washed 3 times with DMEM and finally 1 ml DMEM, 2% FCS was added and the plates were left to incubate for 96 hours (4 days) at 37°C, 5% CO_2 These infections were stopped by freezing the plates at -80°C ready for titration analysis

2 Titration analysis (immunostaining with a Vaccinia virus specific antibody)

For titration of amount of virus test cells (CEF) were seeded on 96-well-plates in RPMI (Gibco, Cat No 61870-010), 7% FCS, 1% antibiotic/ antimycotic (Gibco, Cat No 15240-062) at a concentration of 1 x 10^4 cells/well and incubated over night at 37°C, 5% CO_2 The 6-well-plates containing the infection experiments were frozen/thawed 3 times and dilutions of 10^{-1} to 10^{-12} were prepared using RPMI growth medium Virus dilutions were distributed onto test cells and incubated for five days at 37°C, 5% CO_2 to allow CPE (cytopathic effect) development Test cells were fixed (Aceton/Methanol 1 1) for 10 min, washed with PBS and incubated with polyclonal Vaccinia virus specific antibody (Quartett Berlin, Cat No 9503-2057) at a 1 1000 dilution in incubation buffer for one hour at RT After washing twice with PBS (Gibco, Cat No 20012-019) the HPR-coupled anti-rabbit antibody (Promega Mannheim, Cat No W4011) was added at a 1 1000 dilution in incubation buffer (PBS containing 3% FCS) for one hour at RT Cells were again washed twice with PBS and incubated with staining solution (10 ml PBS + 200 µl saturated solution of o-dianisidine in 100% ethanol + 15 µl H_2O_2 freshly prepared) until brown spots were visible (two hours) Staining solution was removed and PBS was added to stop staining reaction Every well showing a brown spot was marked as positive for CPE

and titer was calculated using the formula of Kaerber ($TCID_{50}$ based assay) (Kaerber, G 1931 Arch Exp Pathol Pharmakol 162, 480)

The viruses were used to infect duplicate sets of CEF and BHK, which were expected to be permissive for MVA, and on the other hand CV-1, Vero, Hela, 143B and HaCat which were expected to be non-permissive for MVA, at a low multiplicity of infection, i e , 0 05 infectious units per cell (5×10^4 $TCID_{50}$) After this, the virus inoculum was removed and the cells washed three time to remove any remaining unabsorbed viruses Infections were left for a total of 4 days where viral extracts were prepared and then titred on CEF cells

It was shown that all viruses amplified well in CEF cells (Chicken embryo fibroblasts) as expected since this is a permissive cell line for all MVAs Additionally, it was shown that all viruses amplified well in BHK (Hamster kidney cell line) MVA-Vero performed the best, since BHK is a permissive cell line

Concerning replication in Vero cells (Monkey kidney cell line) MVA-Vero amplified well as expected namely 1000 fold above Input MVA-HLR and also MVA-575 amplified well with 33 fold and 10 fold increase above Input, respectively Only MVA-BN was found to not amplified as well in these cells as compared to the others, namely only 2-fold increase above Input

Also concerning replication in CV1 cells (Monkey kidney cell line) it was found, that MVA-BN is highly attenuated in this cell line It showed a 200-fold decrease below Input Also MVA-575 did not amplify above the Input level also showed a slightly negative amplification, namely 16-fold decrease below Input MVA-HLR amplified the best with

30-fold increase above Input, followed by MVA-Vero with 5-fold increase above Input

Most interesting is to compare the growth kinetics of the various viruses in human cell lines Regarding reproductive replication in 143B cells (human bone cancer cell line) it was shown that MVA-Vero was the only one to show amplification above Input (3-fold increase) All other viruses did not amplify above Input but there was a big difference between the MVA-HLR and both MVA-BN and MVA-575 MVA-HLR was "borderline" (1 fold decrease below Input), were as MVA-BN shows the greatest attenuation (300 fold decrease below Input) followed by MVA-575 (59 fold decrease below Input) To summarize MVA-BN is superior regarding attenuation in human 143B cells

Furthermore, concerning replication in HeLa cells (human cervix cancer cells) it was shown that MVA-HLR amplified well in this cell line, and even better than it did in the permissive BHK cells (Hela = 125 fold increase above Input BHK = 88 fold increase above Input) MVA-Vero also amplified in this cell line (27 fold increase above Input)

However, MVA-BN and also to a lesser extend MVA-575 were attenuated in these cell lines (MVA-BN = 29 fold decrease below Input and MVA-575 = 6 fold decrease below Input)

Concerning the replication in HaCat cells (human keratinocyte cell line) it was shown that MVA-HLR amplified well in this cell line (55-fold increase above Input) Both MVA-Vero adapted and MVA-575 showed amplification in this cell line (1 2 and 1 1 fold increase above Input respectively) However, MVA-BN and derivatives thereof were the only to demonstrate attenuation (5-fold decrease below Input)

In conclusion it can be stated that MVA-BN is the most attenuated virus strain in this group of virus MVA-BN demonstrates to be extremely attenuated in human cell lines by showing an amplification ratio of 0 05 to 0 2 in Human embryo kidney cells (293 ECACC No 85120602), it shows further an amplification ratio of about 0 0 in 143B cells an amplification ratio of about 0 04 in HeLa cells an amplification ratio of about 0 22 in HaCat cells Additionally, MVA-BN is showing an amplification ratio of about 0 0 in CV1 cells Only in Vero cells amplification can be observed (ratio of 2 33), however, not to the same extent as it in the permissive cell lines such as BHK and CEF Thus, MVA-BN and derivatives thereof are the only known MVA strain showing an amplification ratio of less than 1 in all of the human cell lines 143B, Hela, HaCat and 293

MVA-575 shows a similar profile as MVA-BN but is not as attenuated as MVA-BN

MVA-HLR amplified well in all cell lines tested (except for 143B cells), it thus can be regarded as replication competent in all cell lines tested with exception in 143B cells In one case it even amplified better in a human cell line (HeLa) than in a permissive cell line (BHK)

MVA-Vero does show amplification in all cell lines but to a lesser extent than demonstrated by MVA-HLR (ignoring the 143B result) Nevertheless it cannot be considered as being in the same "class", with regards to attenuation, as MVA-BN or MVA-575

3 Replication in vivo

Given that some MVA strains clearly replicate *in vitro* the ability of different MVA strains to replicate *in vivo* were

examined using a transgenic mouse model AGR129 This mouse strain has gene targeted disruptions in the IFN receptor type I (IFN-α/β) and type II (IFN-γ) genes and in *RAG* Due to these disruptions the mice have no IFN system and are incapable of producing mature B and T cells and as such are severely immune compromised and highly susceptible to a replicating virus Groups of six mice were immunised (i p) with 10^7 pfu of MVA-BN, MVA-HLR or MVA 572 (used in 120,000 people in Germany) and monitored daily for clinical signs All mice vaccinated with MVA-HLR or MVA-572 died within 28 and 60 days, respectively At necropsy there were general signs of a severe viral infection in the majority of organs and by a standard titration assay MVA (10^8 IU (Infectious Unit = $TCID_{50}$) was recovered from the ovaries In contrast, mice vaccinated with the same dose of MVA-BN (corresponding to the deposited strain ECACC V00083008) survived for more than 90 days and no MVA could be recovered from organs or tissues

When taken together the data from the *in vitro* and *in vivo* studies clearly demonstrate that MVA-BN is more highly attenuated than the parental and commercial MVA-HLR strain

4 Different strains of MVA differ in their ability to stimulate the immune response

Replication competent strains of Vaccinia induce potent immune responses in mice and at high doses are lethal Although MVA are highly attenuated and have a reduced ability to replicate on mammalian cells, there are differences in the attenuation between different strains of MVA Indeed, MVA-BN appears to be more attenuated than other MVA strains, even the parental strain MVA-575 To determine whether this difference in attenuation affects the efficacy of MVA to induce protective immune responses,

different doses of MVA-BN and MVA-575 were compared in a lethal Vaccinia challenge model The levels of protection were measured by a reduction in ovary Vaccinia titers determined 4 days post challenge, as this allowed a quantitative assessment of different doses and strains of MVA

5 Lethal Challenge Model

Specific pathogen-free 6-8-week-old female BALB/c ($H\text{-}2^d$) mice (n=5) were immunized (i p) with different doses (10^2, 10^4 or 10^6 $TCID_{50}$/ml) of either MVA-BN or MVA-575 MVA-BN and MVA-575 had been propagated on CEF cells, and had been sucrose purified and formulated in Tris pH 7 4 Three weeks later the mice received a boost of the same dose and strain of MVA, which was followed two weeks later by a lethal challenge (i p) with a replication competent strain of Vaccinia As replication competent Vaccinia virus (abbreviated as "rVV") either the strain WR-L929 TK+ or the strain IHD-J was used Control mice received a placebo vaccine The protection was measured by the reduction in ovary titers determined 4 days post challenge by standard plaque assay For this the mice were sacrificed on day 4 post the challenge and the ovaries were removed, homogenized in PBS (1ml) and viral titers determined by standard plaque assay using VERO cells (Thomson *et a* , 1998, J Immunol 160 1717)

Mice vaccinated with two immunizations of either 10^4 or 10^6 $TCID_{50}$/ml of MVA-BN or MVA-575 were completely protected as judged by a 100% reduction in ovary rVV titers 4 days post challenge The challenge virus was cleared However, differences in the levels of protection afforded by MVA-BN or MVA-575 were observed at lower doses Mice that received two immunizations of 10^2 $TCID_{50}$/ml of MVA 575 failed to be

protected as judged by the high ovary rVV titers (mean 3 7 $x10^7$ pfu +/- 2 11 $x10^7$) In contrast, mice vaccinated with the same dose of MVA-BN induced a significant reduction (96%) in ovary rVV titers (mean 0 21 $x10^7$ pfu +/-0 287 $x10^7$) The control mice that received a placebo vaccine had a mean viral titer of 5 11 $x10^7$ pfu (+/- 3 59 $x10^7$)

Both strains of MVA induce protective immune responses in mice against a lethal rVV challenge Although both strains of MVA are equally efficient at higher doses, differences in their efficacy are clearly evident at sub-optimal doses MVA-BN is more potent than its parent strain MVA-575 at inducing a protective immune response, which may be related to the increased attenuation of MVA-BN compared to MVA-575

SEQUENCE LISTING

<110> Bavarian Nordic A/S
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C L A I M S

1 A recombinant poxvirus comprising at least two heterologous genes, wherein each of the heterologous genes is inserted into a different insertion site of the viral genome and wherein the heterologous genes are homologous among each other

2 The recombinant poxvirus according to claim 1, wherein the homologous genes have a homology of at least 50% in the coding sequence

3 The recombinant poxvirus according to the claims 1 or 2, wherein the homologous genes are derived from a microorganism, preferably from a pathogenic and/or infectious bacterium, fungus or virus

4 The recombinant poxvirus according to claim 3, wherein the homologous genes are derived from two or more members of different strains, clades, variants and/or serotypes of the microorganism, bacterium, fungus or virus

5 The recombinant poxvirus according to claims 3 or 4, wherein the microorganism is a flavivirus, a retrovirus, an enterovirus or a rotavirus

6 The recombinant poxvirus according to claim 5, wherein the flavivirus is a dengue virus and the homologous genes are selected from the PreM, NS1, NS2 or E sequences of different dengue viruses

7 The recombinant poxvirus according to any of the claims 1 to 6, wherein the poxvirus is replication deficient and/or not replicating in human cells

8 The recombinant poxvirus according to any of the claims 1 to 7, wherein the poxvirus is selected from Fowlpoxviruses or derivatives thereof, Canarypoxviruses or derivatives thereof, or attenuated Vaccinia viruses or derivatives thereof

9 The recombinant poxvirus according to claim 8, wherein the attenuated Vaccinia virus is Modified Vaccinia Ankara (MVA), MVA-Vero, MVA-BN or derivatives thereof

10 The recombinant poxvirus according to any of the claims 1 to 9, wherein the insertion sites are selected from naturally occurring deletions in the pox virus genome, non-essential gene loci and/or intergenic regions

Abstract of the Invention

The present invention relates to a recombinant poxvirus vector capable of expressing two or more sequences, which derive from different variants of a microorganism, and which have between each other a homology of 50% or above The invention further relates to a method preparing such recombinant poxvirus and the use of such recombinant poxvirus as medicament or vaccine Additionally, a method for affecting, preferably inducing, an immune response in a living animal, including a human, is provided

Fig 1 ALIGNED SEQUENCES

Reference molecule		PrM4	1 - 573	(573 bps)	Homology	
Sequence	2	PrM3	1 - 573	(573 bps)	66%	
Sequence	3	PrM2	1 - 573	(573 bps)	67%	
Sequence	4	PrM1	1 - 573	(573 bps)	65%	

Alignment type: Global DNA
Parameters: Mismatch 2, Open Gap 4, Extend Gap 1

```
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PrM2  (   1)            t     a     cgc  aa tg a gc tg  cat a   -
PrM1  (   1)    t       aa    a     g    .a  tgt-  cc tgc c  catgc  c g .

PrM4  (  60) caccgtaatggcgttttccttgtcaacaagagatggcgaacccctcatgatagtggcaaa
PrM3  (  60) ag aac c t  t  cca   aa tt  c       a  g  g g      t    gg
PrM2  (  60) a  a  g        ccat  aa c   c ta c  a     a a       c  cagt g
PrM1  (  60)    a ccc       ccatc  a c  cc   gg  a  g  g a         tagc

PrM4  ( 120) acatgaaaggggagacctctcttgtttaagacaacagaggggatcaacaaatgcactct
PrM3  ( 120) ga       a  a.a t c  ac t          g ctct  a       tg     a .
PrM2  ( 120)    a  g aa     a ag   tc       a    gag. t  tg g    tg  t  c .
PrM1  ( 120) g  g     a  a a t a  t    .       ct t.ca  tg      tg    . c..

PrM4  ( 180) cattgccatggacttgggtgaaatgtgtgaggacactgtcacgtataaatgccccctact
PrM3  ( 180)    a        t     a  g        t     g     t  c              ----
PrM2  ( 180)    g         c t       t       a  t  aa       c  g  t  tt t .
PrM1  ( 180) t     a     t g   a  gt a             aa g   c  c        .  gga

PrM4  ( 240) ggtcaataccgaa------cctgaagacattgattgctggtgcaacctcacgtctacatg
PrM3  ( 236) --a  t       gtggag                  c  t              t  a  g    .
PrM2  ( 240) caagc g at . ------  a          a .   t           tct     c  .
PrM1  ( 240) cactg g  g   ------  a  t   g     c  t          tgc     gag

PrM4  ( 294) ggtcatgtatgggacatgcacccagagcggagaacggagacgagagaagcgctcagtagc
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PrM2  ( 294)    a ct      .   g  t   acc ca        ac    a   .a  aa a     g
PrM1  ( 294)  . g cc      .a     tt t  a ct  t     acc       c  a  t .c  c .

PrM4  ( 354) tttaacaccacattcaggaatgggattggaaacaagagctgagacatggatgtcatcgga
PrM3  ( 354) g   g t  c . gtc  c       c     c    c ca  c a  c         gg t
PrM2  ( 354) ac cgtt      gtg          c     g    c  a     a             a
PrM1  ( 354) ac gg       .cgt   gc t  tc a          a c  a  g         c  t

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PrM2  ( 414)       c    .a     c       a t  a ct      ct g   c t    g c  ta
PrM1  ( 414)    c        a  aata  a a   g    c    gct  g   c.    g      a

PrM4  ( 474) gctcttggcaggatttatggcttatatgattgggcaaacaggaatccagcgaactgtctt
PrM3  ( 474) ca ac a  cct    c t  cc  tac  a  cactt cttg c    aa gtg  ta
PrM2  ( 474) ca aa       c a cc    a  c cc  a  aacg   catt    aa  g cc ga
PrM1  ( 474)  g ga a  cctc. cc a  ac  gcc  a  aac t catc c    aa ggga .a

PrM4  ( 534) ctttgtcctaatgatgctggtcgccccatcctacggatga
PrM3  ( 534)    ca a   t a          ta    .   atgac
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PrM1  ( 534)    ca tt gc           aa t      atg cc
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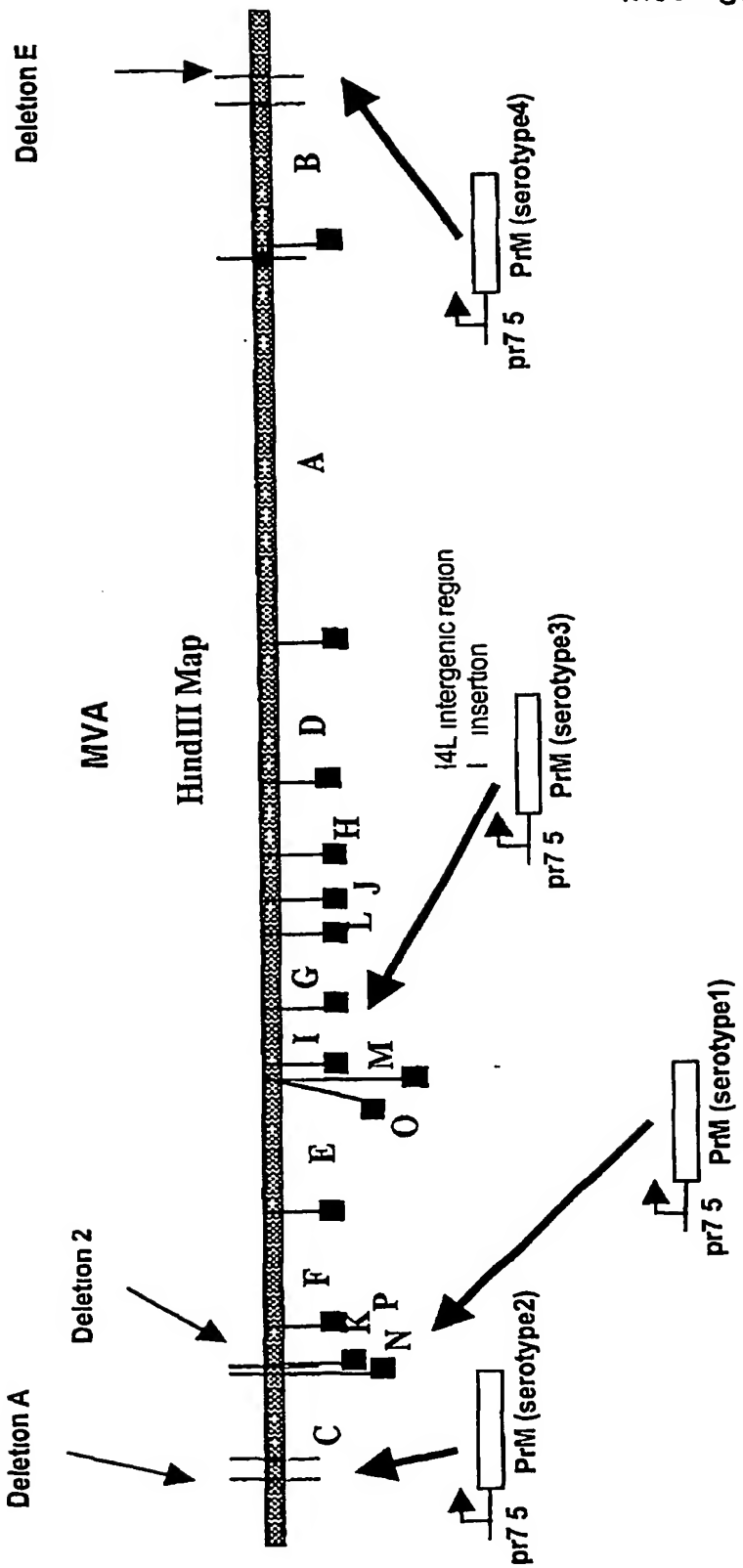

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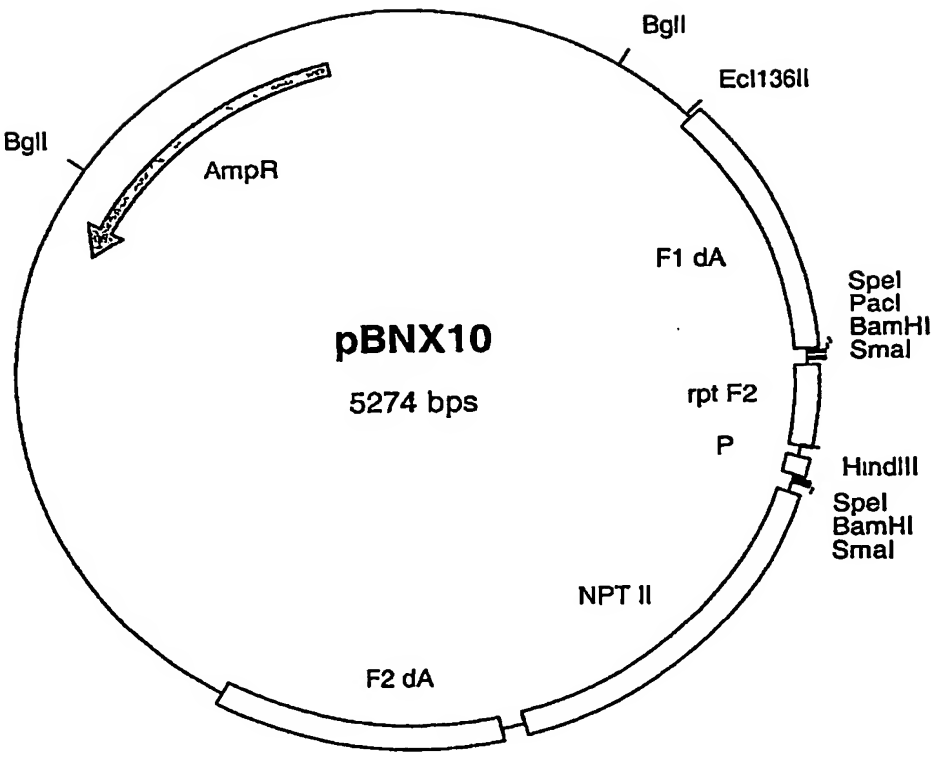

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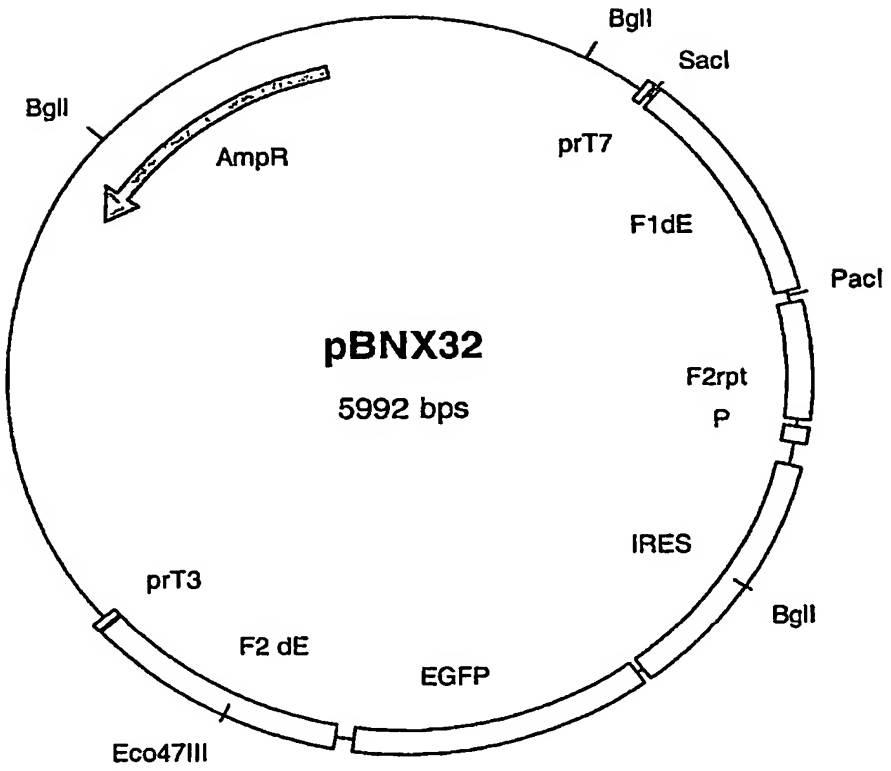

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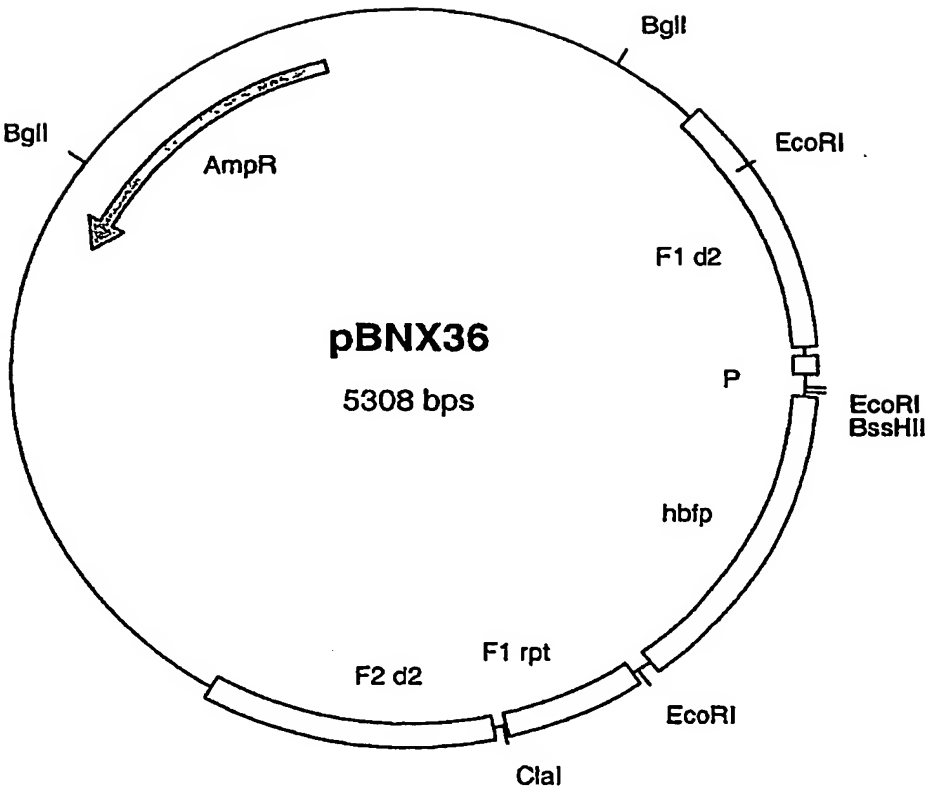

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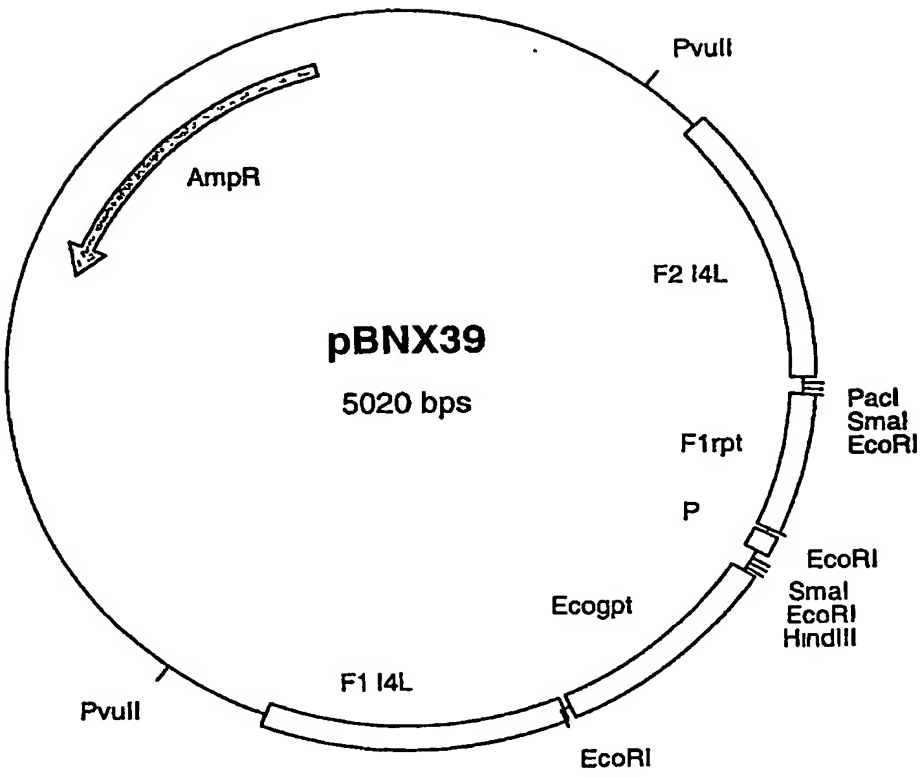

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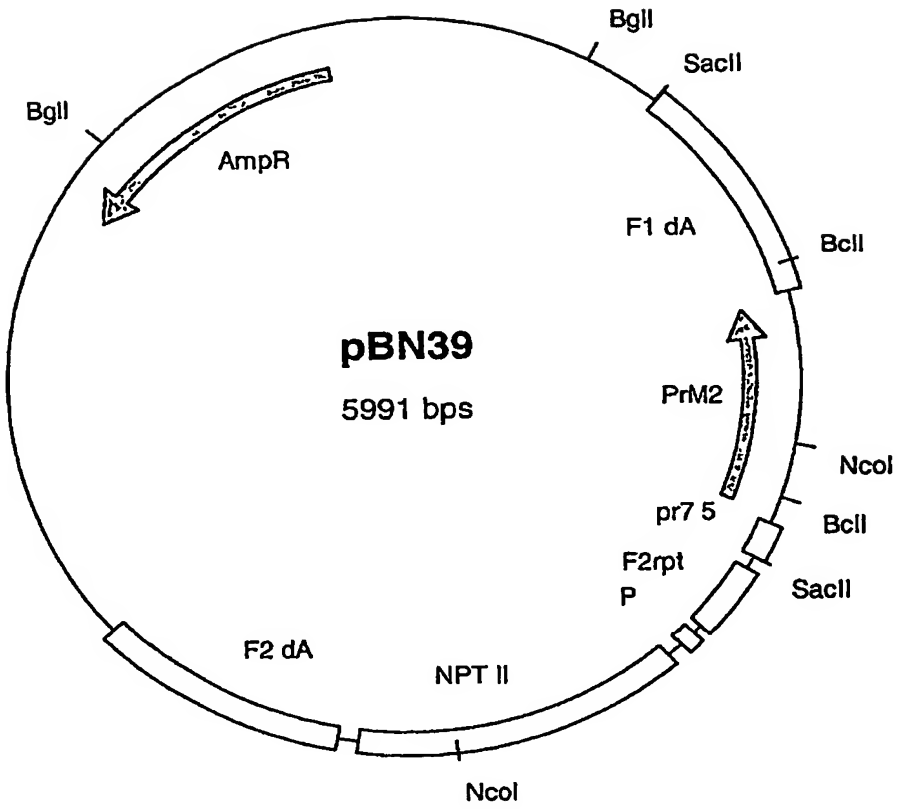

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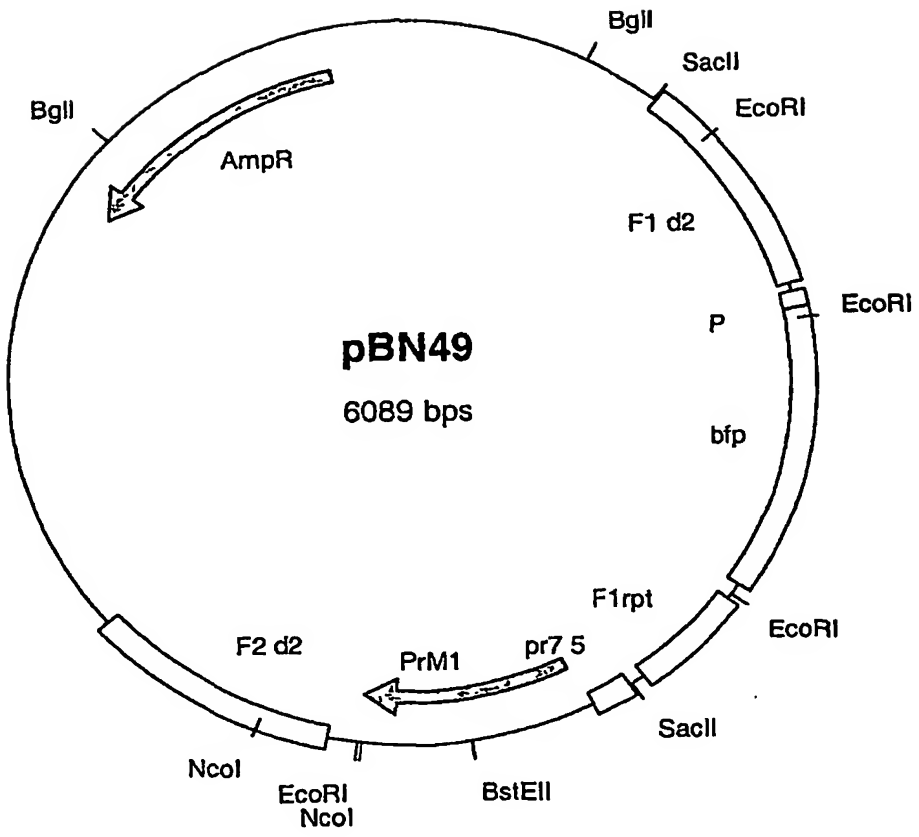

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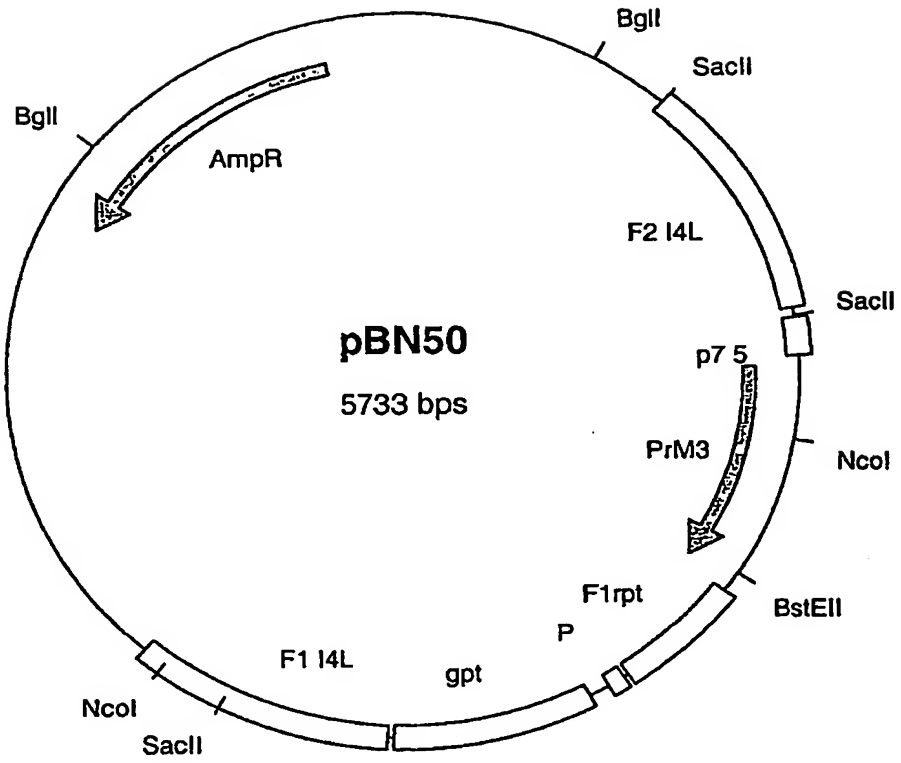

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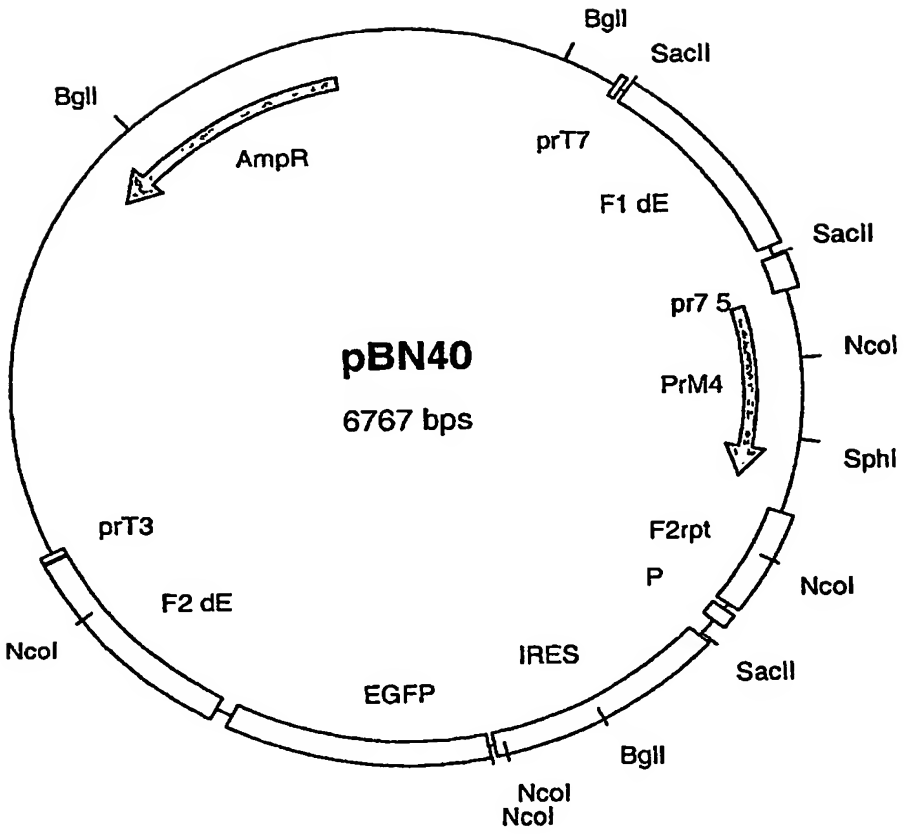

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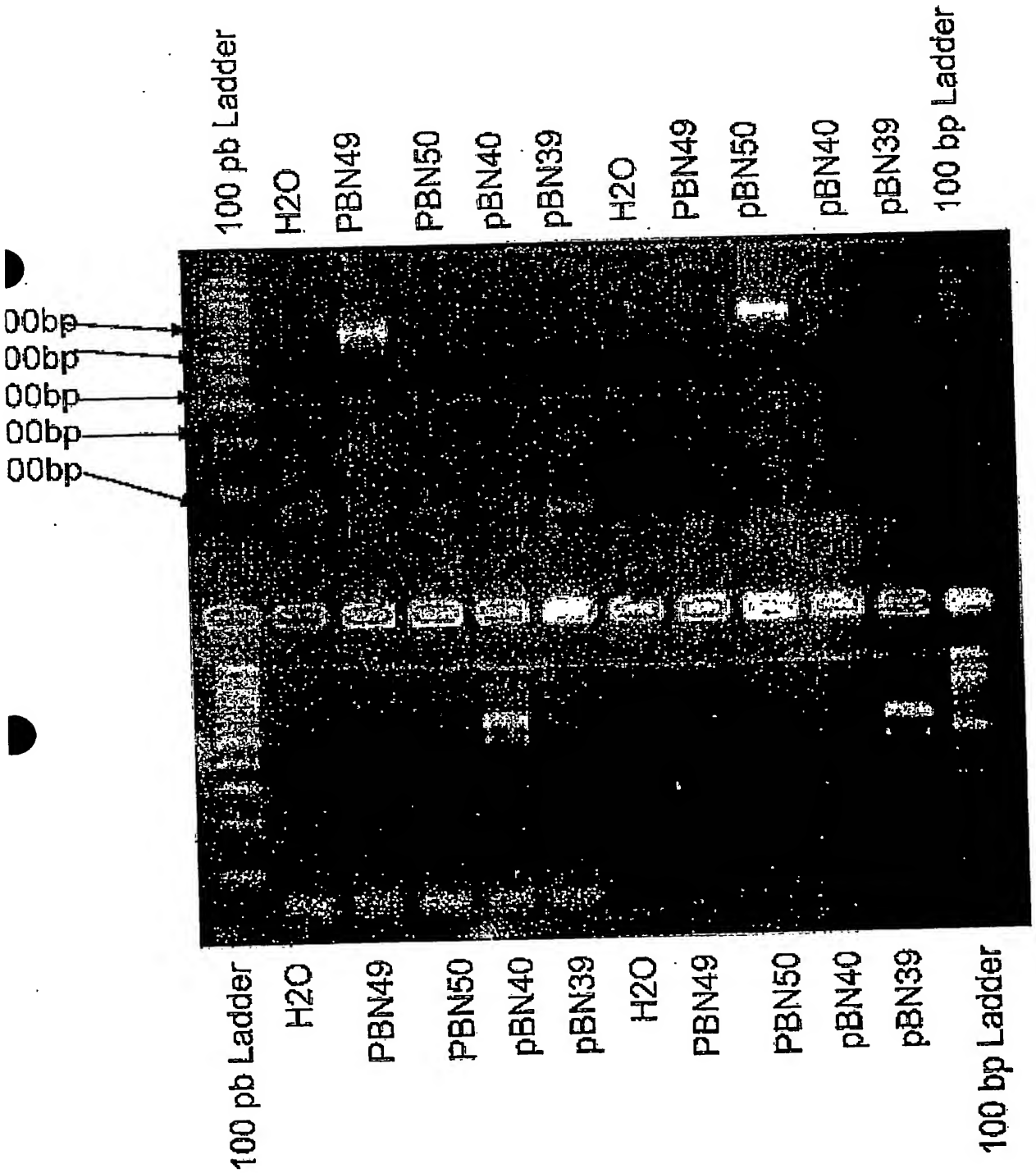


Patent- og Varemærkestyrelsen
Helgeshøj Allé 81
2630 Taastrup

København 16 maj 2002

Vor ref : BN46DK

Indlevering af ansøgning "Expression of homologous sequences"

Hermed vedlagt en patentansøgning med titlen " Expression of homologous sequences"

Patentansøgningen ønskes nyhedsundersøgt på baggrund af de engelsksprogede dokumenter

Vedlagt er "Oplysning om deponering af biologisk materiale" inklusiv yderligere angivelser,
en sekvensliste på diskette og
et overdragelsesdokument opfinderne til fra Bavarian Nordic GmbH inklusiv den yderligere overdragelsen til Bavarian Nordic A/S

Med venlig hilsen

Charlotte Utermøhl Lund

BAVARIAN NORDIC

Bavarian Nordic A/S
Vesterbrogade 149, DK 1620 Copenhagen V, Denmark, Phone +45 33 26 83 83, Fax +45 33 26 83 80
www.bavarian-nordic.com A/S Reg No 208 618 VAT No DK 16 27 11 87

Oplysning om deponering af biologisk materiale

Ansøgningen omfatter følgende deponeringer i henhold til Patentlovens § 8a stk 1 eller Brugsmodellovens § 8, stk 1

Patent- og
Varemærkestyrelsen
Erhvervsministeriet

Helgeshøj Allé 81
2630 Taastrup

Tlf 43 50 80 00
Fax 43 50 80 01
Postgiro 8 989 923
E-post pvs@dkpto.dk
www.dkpto.dk

A Identifikation af deponeringer

1 Vedrørende det på side 13 linie 1-4 i beskrivelsen omtalte biologiske materiale

Deponeringsinstitutionens navn
European Collection of Cell Cultures, CAMR

Deponeringsinstitutionens adresse (inklusive postnummer og land)
Salisbury, Wiltshire SP4 OJG, United Kingdom, Tel + 44 19 80 61 25 12

Dato for deponering 30 August 2000 Løbenummer V00083008

2 Vedrørende det på side 12 linie 15-17 i beskrivelsen omtalte biologiske materiale

Deponeringsinstitutionens navn
European Collection of Cell Cultures, CAMR

Deponeringsinstitutionens adresse (inklusive postnummer og land)
Salisbury, Wiltshire SP4 OJG, United Kingdom, Tel + 44 19 80 61 25 12

Dato for deponering 7 Dezember 2000 Løbenummer V00120707

3 Vedrørende det på side 12 linie 19-27 i beskrivelsen omtalte biologiske materiale

Deponeringsinstitutionens navn
European Collection of Cell Cultures, CAMR

Deponeringsinstitutionens adresse (inklusive postnummer og land)
Salisbury, Wiltshire SP4 OJG, United Kingdom, Tel + 44 19 80 61 25 12

Dato for deponering 14 Oktober 1999 Løbenummer V99101431

☐ Yderligere oplysninger på et følgende ark

B Yderligere angivelser, fx om det biologiske materiales farlighed, geografisk oprindelse

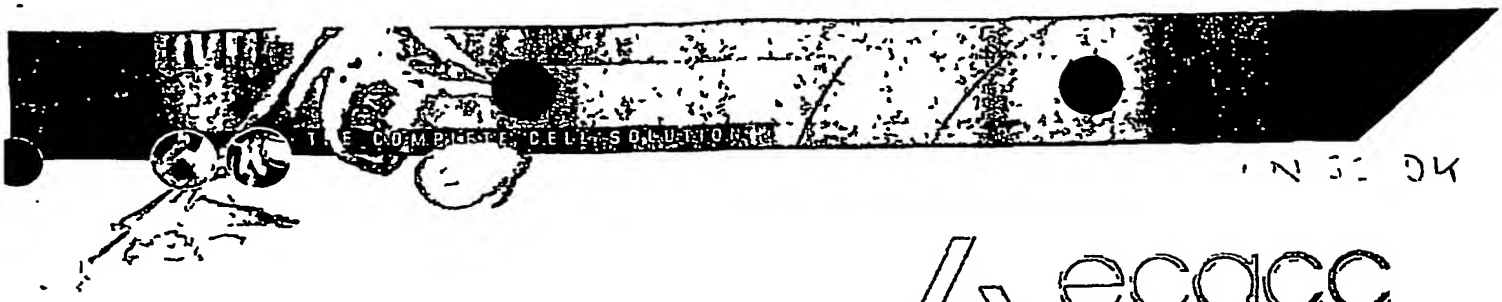
☑ Oplysningerne fortsættes på et vedføjet ark

C ☑ Det begæres at udlevering af en prøve i tiden indtil ansøgningen er fremlagt eller endeligt afgjort uden at være fremlagt kun sker til særlig sagkyndig jfr PL § 22 stk 7 eller BML § 8 stk 2

Dato og underskrift

(Peter Wulff)
Bo Jansen

17 mar00/ls



ecacc
European Collection of Cell Cultures

Centre for Applied Microbiology and Research
&
European Collection of Cell Cultures

This document certifies that Virus
(Deposit Ref V99101431) has been accepted as a patent deposit,
in accordance with
The Budapest Treaty of 1977,
with the European Collection of Cell Cultures on 14TH October 1999

Dr P J Packer
Quality Manager, ECACC

CAMR
European Collection of Cell Cultures CAMR Salisbury Wiltshire SP4 0JG UK
Tel 44 (0) 1980 612512 Fax 44 (0) 1980 611315 Email ecacc@camr.org.uk Web Site ecacc.org.uk

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO
PROF DR DR H C MULT ANTON MAYR
NEILHEIMER STR 1
D-82319 STARNBERG
GERMANY

NAME AND ADDRESS
OF DEPOSITOR

I IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR

VERO-MVA

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY

V99101431

II SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 14TH October 1999 (date of the original deposit)[1]

IV RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and
A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV INTERNATIONAL DEPOSITORY AUTHORITY

Name Dr P J Packer

Address ECACC
CAMR
Porton Down
Salisbury SP4 OJG

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s)

Date 2/3/01 PJPacker

1 Where Rule 6 4(d) applies, such date is the date on which the status of international depositary authority was acquired

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

PROF DR DR H C MULT ANTON MAYR
WEILHEIMER STR 1
D-82319 STARNBERG
GERMANY

VIABILITY STATEMENT
Issued pursant to Rule 10 2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY OF STATEMENT
IS ISSUED

I DEPOSITOR	II IDENTIFICATION OF THE MICROORGANISM
Name PROF DR DR H C MULT ANTON MAYR Address WEILHEIMER STR 1 D-82319 STARNBERG GERMANY	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY V99101431 Date of the deposit or of the transfer[1] 14TH October 1999
II VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on [2] On that date, the said microorganism was [X] [3] viable [ ] [3] no longer viable	

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most relevant date (date of the new deposit or date of the transfer)

2 In the cases referred to in Rule 10 2 (a) (ii) and (iii), refer to the most recent viability test

3 Mark with a cross the applicable box

IV CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]

VERO-MVA - 99101431

THE VIRUS WAS GROWN ON VERO CELLS ACCORDING TO THE DEPOSITORS INSTRUCTIONS THE VIRUS WAS VIABLE PRODUCING CYTOPATHIC EFFECT AFTER 48 HOURS A LITRE OF 6 X 10^6 PLAQUE FORMING UNITS/ML WAS OBTAINED

V INTERNATIONAL DEPOSITARY AUTHORITY

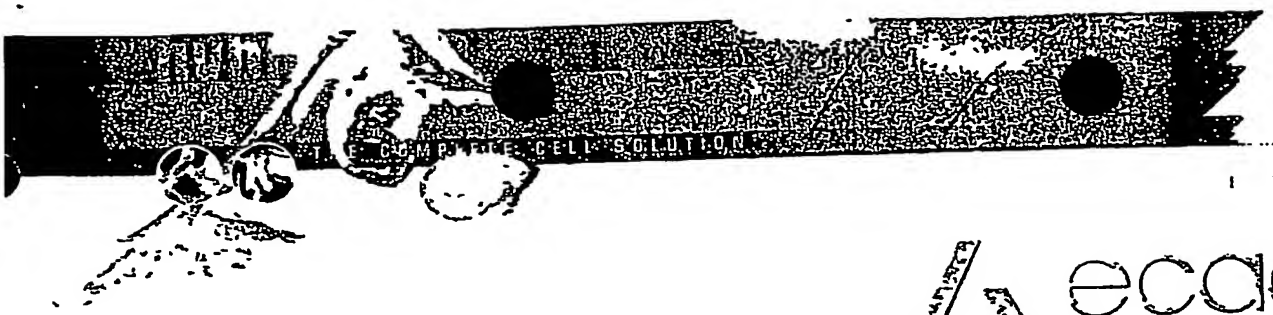
Name Dr P J Packer
ECACC CAMR

Address Porton Down
Salisbury
Wiltshire
SP4 0JG

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s)

Date 8/3/01 PJ Packer

4 Fill in if the information has been requested and if the results of the test were negative



ecacc
European Collection
of Cell Cultures

Centre for Applied Microbiology and Research
&
European Collection of Cell Cultures

This document certifies that Virus
(Deposit Ref V00083008) has been accepted as a patent deposit,
in accordance with
The Budapest Treaty of 1977,
with the European Collection of Cell Cultures on 30TH August 2000

Dr P J Packer
Quality Manager, ECACC

European Collection of Cell Cultures CAMR Salisbury Wiltshire SP4 0JG UK
Tel 44 (0) 1980 612512 Fax 44 (0) 1980 611315 Email ecacc@camr.org.uk Web Site ecacc.org.uk

IV CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]
V00083008 - MVA-BN VIABILITY OF MVA-BN WAS TESTED BY GROWING THE VIRUS ON BHK CELLS AND CALCULATING THE TCD50

V INTERNATIONAL DEPOSITARY AUTHORITY	
Name Dr P J Packer ECACC CAMR Address Porton Down Salisbury Wiltshire SP4 0JG	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) Date 14/12/00 PJPacker

4 Fill in if the information has been requested and if the results of the test were negative

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

VIABILITY STATEMENT
Issued pursant to Rule 10 2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY OF STATEMENT
IS ISSUED

I DEPOSITOR		II IDENTIFICATION OF THE MICROORGANISM
Name	BAVARIAN NORDIC RESEARCH INSTITUTE GMBH	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY V00083008
Address	FRAUNHOFERSTRASSE 18B D-82152 MARTINSRIED GERMANY	Date of the deposit or of the transfer 30TH August 2000

II VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on [1] On that date, the said microorganism was

[X] [3] viable

[ ] [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most relevant date (date of the new deposit or date of the transfer)

2 In the cases referred to in Rule 10 2 (a) (ii) and (iii), refer to the most recent viability test

3 Mark with a cross the applicable box

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

NAME AND ADDRESS
OF DEPOSITOR

I IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR MVA-BN	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY V00083008

II SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 30TH August 2000 (date of the original deposit)[1]

IV RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and
A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV INTERNATIONAL DEPOSITORY AUTHORITY

Name Dr P J Packer

Address ECACC
CAMR
Porton Down
Salisbury SP4 OJG

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s)

Date 14/12/00

1 Where Rule 6 4(d) applies, such date is the date on which the status of international depositary authority was acquired

Form BP/4 (sole page)

1991

Certificate of Analysis

Product Description MVA-BN
Accession Number 00083008

Test Description The Detection of Mycoplasma by Isolation on Mycoplasma Pig Serum Agar and in Mycoplasma Horse Serum Broth
SOP QC/MYCO/01/02

Acceptance Criterion/Specification: All positive controls (*M pneumoniae & M orale*) must show evidence of mycoplasma by typical colony formation on agar plates Broths are subcultured onto Mycoplasma Pig Serum Agar where evidence of mycoplasma by typical colony formation is evaluated All negative control agar plates must show no evidence of microbial growth
The criteria for a positive test result is evidence of mycoplasma by typical colony formation on agar A negative result will show no such evidence

Test Number 21487

Date. 27/11/00

Result.

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Test Description: Detection of Mycoplasma using a Vero indicator cell line and Hoechst 33258 fluorescent detection system
SOP QC/MYCO/07/05

Acceptance Criterion/Specification: The Vero cells in the negative control are clearly seen as fluorescing nuclei with no cytoplasmic fluorescence Positive control (*M orale*) must show evidence of mycoplasma as fluorescing nuclei plus extra nuclear fluorescence of mycoplasma DNA Positive test results appear as extra nuclear fluorescence of mycoplasma DNA Negative results show no cytoplasmic fluorescence

Test Number 21487

Date 27/11/00

Result:

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Authorised by PSPaul . **ECACC, Head of Quality** 4/12/00 . **Date**

Certificate of Analysis

Product Description MVA-BN
Accession Number 00083008

Test Description Detection of bacteria and fungi by isolation on Tryptone Soya Broth (TSB) and in Fluid Thioglycollate Medium (FTGM) SOP QC/BF/01/02

Acceptance Criterion/Specification All positive controls (*Bacillus subtilus, Clostridium sporogenes* and *Candida albicans*) show evidence of microbial growth (turbidity) and the negative controls show no evidence of microbial growth (clear)
The criteria for a positive test is turbidity in any of the test broths All broths should be clear for negative test result

Test Number 21487
Date 27/11/00
Result

Positive Control	Positive
Negative Control	Negative
Test Result.	Negative
Overall Result	PASS

Test Description. Determination of $TCID_{50}$ of cytopathic Virus titration (SOP ECACC/055) Cell

Acceptance Criterion/Specification/Criteria Negative controls should show no sign of Cytopathic effects The Test Sample is serially diluted into in 4 wells of indicator cell lines for each dilution Cytopathic effects indicate that virus is present Virus titre is calculated using the below equation where x is the value obtained from a standard $TCID_{50}$ Table as a result of the distribution of the wells displaying less than 4 positive wells per dilution, and y is the value of the highest dilution where all 4 wells are positive

$$TCID_{50} = \frac{1}{y} \times 10^{1+x}$$

Date. 01/12/00

Result:

Indicator Cell Line	BHK21 (Clone 13)
Negative Control	NO CPE
Test Sample	CPE
Distribution of less that 4 positive wells	4, 4, 4, 3, 0
X	1 25
Y	10^{3}

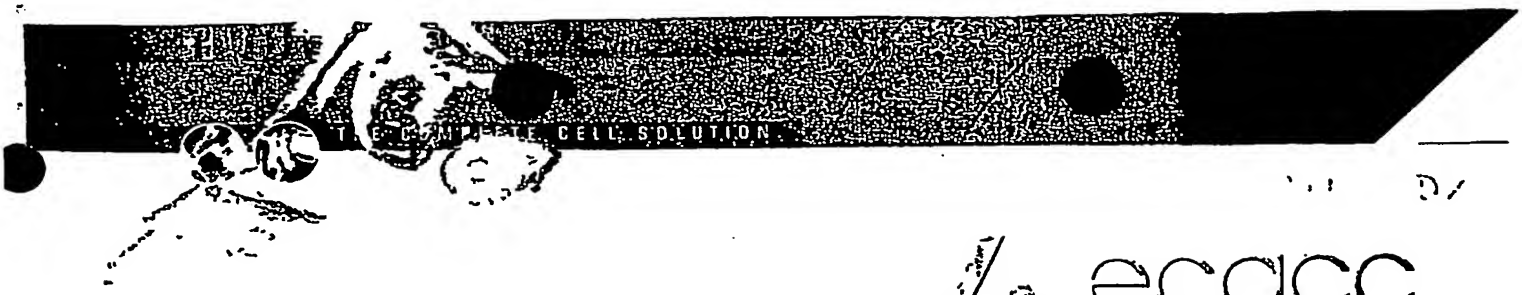
$$TCID_{50} = \frac{1}{10^{7}} \times 10^{1+0125}$$

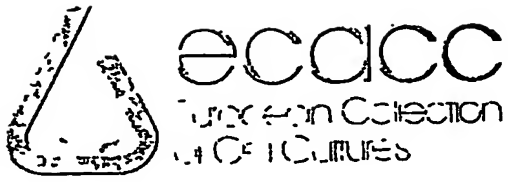
$$= 10^{5\,25}$$

Overall Result Virus Present

*** End of Certificate***

Authorised by ..... ECACC, Head of Quality 4/12/00 **Date**





Centre for Applied Microbiology and Research
&
European Collection of Cell Cultures

This document certifies that Virus
(Deposit Ref V00120707) has been accepted as a patent deposit,
in accordance with
The Budapest Treaty of 1977,
with the European Collection of Cell Cultures on 7TH December 2000

Dr P J Packer
Quality Manager, ECACC

European Collection of Cell Cultures CAMR, Salisbury Wiltshire SP4 0JG UK
Tel 44 (0) 1980 612512 Fax 44 (0) 1980 611315 Email ecacc@camr.org.uk Web Site ecacc.org.uk

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

NAME AND ADDRESS
OF DEPOSITOR

I IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR MVA-575	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY V00120707

II SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 7TH December 2000 (date of the original deposit)[1]

IV RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and
A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV INTERNATIONAL DEPOSITORY AUTHORITY

Name Dr P J Packer

Address ECACC
CAMR
Porton Down
Salisbury SP4 0JG

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s)

Date

1 Where Rule 6 4(d) applies, such date is the date on which the status of international depositary authority was acquired

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

VIABILITY STATEMENT
Issued pursant to Rule 10 2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY OF STATEMENT
IS ISSUED

I DEPOSITOR	II IDENTIFICATION OF THE MICROORGANISM
Name BAVARIAN NORDIC RESEARCH INSTITUTE GMBH Address FRAUNHOFERSTRASSE 18B D-82152 MARTINSRIED GERMANY	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY 00120707 Date of the deposit or of the transfer[1] 7TH December 2000

II VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on [2]. On that date, the said microorganism was

☐ [3] viable

☐ [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most relevant date (date of the new deposit or date of the transfer)

2 In the cases referred to in Rule 10 2 (a) (ii) and (iii), refer to the most recent viability test

3 Mark with a cross the applicable box

IV CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]	
MVA-575 - V00120707 THIS VIRUS WAS TITRATED ON BHK CELLS $TCID_{50} = 10^{8.3}$	
V INTERNATIONAL DEPOSITARY AUTHORITY	
Name Dr P J Packer ECACC CAMR Address Porton Down Salisbury Wiltshire SP4 0JG	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) Date 23/3/01

4 Fill in if the information has been requested and if the results of the test were negative

Certificate of Analysis

Product Description MVA-575
Accession Number 00120707

Test Description Determination of $TCID_{50}$ of cytopathic Virus titration (SOP ECACC/055) Cell

Acceptance Criterion/Specification/Criteria Negative controls should show no sign of Cytopathic effects The Test Sample is serially diluted into in 4 wells of indicator cell lines for each dilution Cytopathic effects indicate that virus is present Virus titre is calculated using the below equation where x is the value obtained from a standard $TCID_{50}$ Table as a result of the distribution of the wells displaying less than 4 positive wells per dilution, and y is the value of the highest dilution where all 4 wells are positive

$$TCID_{50} = \frac{1}{y} \times 10^{1+x}$$

Date 19/01/01

Result

Indicator Cell Line	BHK 21 CLONE 13
Negative Control	NO CPE
Test Sample	CPE
Distribution of less that 4 positive wells	4, 4, 0
X	0 50
Y	10^5

$$TCID_{50} = \frac{1}{10^5} \times 10^{1+0.50}$$

$$= 10^{6.5}$$

Overall Result Virus Present

Test Description The Detection of Mycoplasma by Isolation on Mycoplasma Pig Serum Agar and in Mycoplasma Horse Serum Broth
SOP QC/MYCO/01/02

Acceptance Criterion/Specification All positive controls (*M pneumoniae & M orale*) must show evidence of mycoplasma by typical colony formation on agar plates Broths are subcultured onto Mycoplasma Pig Serum Agar where evidence of mycoplasma by typical colony formation is evaluated All negative control agar plates must show no evidence of microbial growth
The criteria for a positive test result is evidence of mycoplasma by typical colony formation on agar A negative result will show no such evidence

Test Number 21702
Date 12/02/01
Result

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Authorised by [signature] **ECACC, Head of Quality** 5/3/01 **Date**

Certificate of Analysis

Product Description MVA-575
Accession Number 00120707

Test Description Detection of Mycoplasma using a Vero indicator cell line and Hoechst 33258 fluorescent detection system
SOP QC/MYCO/07/05

Acceptance Criterion/Specification The Vero cells in the negative control are clearly seen as fluorescing nuclei with no cytoplasmic fluorescence Positive control (*M orale*) must show evidence of mycoplasma as fluorescing nuclei plus extra nuclear fluorescence of mycoplasma DNA Positive test results appear as extra nuclear fluorescence of mycoplasma DNA Negative results show no cytoplasmic fluorescence

Test Number 21702

Date 12/02/01

Result

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Test Description Detection of bacteria and fungi by isolation on Tryptone Soya Broth (TSB) and in Fluid Thioglycollate Medium (FTGM) SOP QC/BF/01/02

Acceptance Criterion/Specification All positive controls (*Bacillus subtilus, Clostridium sporogenes* and *Candida albicans*) show evidence of microbial growth (turbidity) and the negative controls show no evidence of microbial growth (clear)
The criteria for a positive test is turbidity in any of the test broths All broths should be clear for negative test result

Test Number 21702

Date 12/02/01

Result

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Authorised by [signature] **ECACC, Head of Quality** 5/3/01 **Date**